

**Formation of
Proinsulin and
Bifunctional Enzymes by
Genetically Engineered
Bacteria**

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Lund 1986

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"En bok som inte är värd att läsas två gånger
är inte värd att läsas en gång".

X.X.

"En bok som inte är värd att läsas en gång
borde istället läsas två gånger".

S.B.

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LIST OF PAPERS

This thesis is based on the following papers, which are referred to by Roman numerals in the text :

- I. Ligation of Restriction Endonuclease-Generated DNA Fragments Using Immobilized T4 DNA Ligase.
L. Bülow and K. Mosbach
Biochem. Biophys. Res. Comm. 107, 458-464 (1982)
- II. Formation of Proinsulin by Immobilized *Bacillus subtilis*.
K. Mosbach, S. Birnbaum, K. Hardy, J. Davies and L. Bülow
Nature 302, 543-545 (1983)
- III. Automated Thermometric Enzyme Immunoassay of Human Proinsulin Produced by *Escherichia coli*.
S. Birnbaum, L. Bülow, K. Hardy, B. Danielsson and K. Mosbach
Anal Biochem. in press
- IV. Preparation of a Soluble Bifunctional Enzyme by Gene Fusion.
L. Bülow, P. Ljungcrantz and K. Mosbach
Bio/Technology 3, 821-823 (1985)
- V. Characterization of an Artificial Bifunctional Enzyme, Beta-Galactosidase/Galactokinase, Prepared by Gene Fusion.
L. Bülow
submitted for publication
- VI. The Expression in *Escherichia coli* of a Polymeric Gene Coding for an Esterase Mimic Catalyzing the Hydrolysis of p-Nitrophenyl Esters.
L. Bülow and K. Mosbach
submitted for publication

1. INTRODUCTION

A living cell is a protein factory. It synthesizes the enzymes and proteins necessary for its maintenance. Depending on among other things the supply of nutrients and kind of cell different proteins are formed, following the instructions encoded in the DNA.

With the development of genetic engineering it has become possible to alter those instructions, especially in bacteria but in recent years also in eukaryotic cells. Thereby can cells be created that are able to synthesize new proteins. Thus, they contain together with their own genes, a foreign gene, for instance of human origin. If the inserted gene is a structural gene, a culture of the manipulated bacteria, which can be grown at a low cost, will serve as an efficient factory for production of the protein in question.

The interest in genetic engineering has experienced a very rapid growth: in 1976 Chemical Abstracts listed only 10 entries on "genetic engineering" while in 1985 there were 280 (Figure 1). During these years a number of basic recombinant DNA techniques have been developed, for example DNA sequencing, chemical synthesis of oligonucleotides and various blotting procedures. However, our present-day DNA technology is totally dependent on the employment of enzymes for cleaving and ligating DNA. In Chapter 2 (Paper I), the use of immobilized enzymes for DNA manipulations is evaluated.

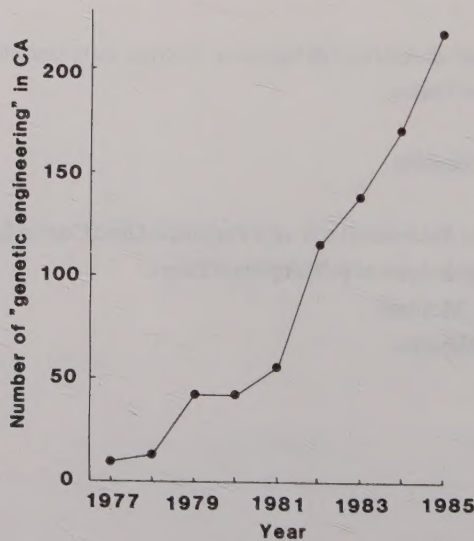


Figure 1. The development of genetic engineering as illustrated by the number of entries on "genetic engineering" in Chemical Abstracts.

Manipulation of the bacterial genome to produce bacteria capable of forming pharmacologically useful peptides has since the beginning of recombinant DNA technology been an area of intense research activity. Using gene cloning, bacteria can be engineered to produce proteins normally found only in higher eukaryotes, thus making many of these proteins commercially available for the first time or secure supply if scarce. Indeed, some human proteins produced in bacteria, such as human growth hormone and insulin, are already on the market. In Chapter 3 (Paper II), a production protocol for proinsulin using immobilized *Bacillus subtilis* is described. Genetically engineered bacteria, almost exclusively in the form of *Escherichia coli*, have usually been grown batchwise in a fermentor. This procedure suffers from several disadvantages. Large cell masses have to be handled, the cells must be opened and protein purification is often complex. The use of *B. subtilis* as a recipient of the inserted gene is highly advantageous because it can be tailored to secrete its cloned gene product to the growth medium. In combination with its immobilization (entrapment), a simple production scheme can be designed. However, in the immobilized form a prerequisite is the inhibition of cell growth. Out of a number of potential procedures of carrying out such an inhibition, addition of certain antibiotics in the growth medium, such as novobiocin and nalidixic acid which inhibit DNA replication, has proved to be a simple method of fulfilling this requirement. Such an addition should also offer an interesting method of controlling cell division of bacteria free in suspension.

One of the most exciting aspects of genetic engineering is that it is not restricted to the use of only existing genes. The naturally occurring genes can be modified and thereby can new proteins be created, a technique which has been dubbed **protein engineering**. Protein engineering comprises a wide variety of recombinant DNA techniques:

- site-directed mutagenesis
- gene fusion
- *de novo* protein design

The simplest example to generate novel proteins involves redesigning of a protein by site-directed mutagenesis. Such amino acid changes can be made by mismatch primer mutagenesis or cassette mutagenesis. The investigations of e.g. Wilkinson *et al.* (1984) and Wells *et al.* (1985) have shown the power of this approach.

When a cell is studied, its metabolism can be characterized by the action of enzyme sequences. Usually, each step in a metabolic pathway is catalyzed by an enzyme existing specifically for that

purpose. Thus, living cells contain thousands of different enzymes where each individual enzyme *in vivo* is highly integrated in the metabolic machinery. Furthermore, from the emerging views on enzymic infrastructure, it is clear that there is a spatial organization of the enzymes in a metabolic pathway, entailing a wide scope of protein complexes from weak protein-protein interactions to strictly organized multienzyme complexes. An extreme evolution of the latter is the bi- or even multifunctional proteins possessing more than one autonomous catalytic or binding site on the same polypeptide chain. These proteins have most probably evolved from smaller proteins through gene fusion (Kirschner and Bisswanger, 1976). Chapter 4 (Paper IV and V) deals with the preparation of an artificial bifunctional enzyme, beta-galactosidase/galactokinase, employing *in vitro* gene fusion. The hybrid enzyme is able to catalyze the hydrolysis of lactose followed by the phosphorylation of galactose. Great interest is taken in various proximity effects such as substrate channeling as well as shifts in pH optima and K_m .

The use of gene fusions is not new. Already since the beginning of genetic engineering such fusions have played an important role mainly for stabilization *in vivo* of eukaryotic proteins in *E. coli* (Itakura *et al.*, 1977), but more recently also for protein purification (Smith *et al.*, 1984 and Nilsson *et al.*, 1985) and stabilization against heat denaturation *in vitro* (Bülow and Mosbach, 1985).

One of the most thrilling challenges to a biochemist is the preparation of a novel enzyme from the ground up. In Chapter 5 (paper VI), an attempt to prepare an esterase/protease mimic using genetic engineering techniques is described. An important technical break-through that make such *de novo* synthesis feasible, is the ability to synthesize oligonucleotides of a defined sequence both rapidly and inexpensively. This is largely due to the development of solid phase synthetic methods used in automated procedures (Narang, 1983). Today, oligonucleotides must be considered standard laboratory reagents. However, the development of artificial enzymes is hampered by our insufficient knowledge of protein folding. X-ray crystallography is a vital tool for determining protein structure and its relationship to the catalytic action of enzymes. Our understanding of the specificity and rate enhancements afforded by enzymes has come about from the three-dimensional structures made available through this technique. However, the determination of the tertiary structure of a protein by these diffraction methods is a tedious procedure. The three-dimensional structure of only about 120 proteins have been solved yet. This in turn entails that the rules governing protein folding is far from being known. Therefore, the design of an artificial protein must focus on mimicing known protein structures. We chose to investigate serin protease mimics because the structure and mechanism of action of these enzymes are one of the most thoroughly studied.

In Chapter 6, some practical applications of the above described papers will be discussed, the major emphasis being devoted to application in analysis.

One of the main goals of this thesis has been to pay attention to the use of genetic engineering techniques in the area of enzyme technology and *vice versa*. Until recently, there has been much of a vacant space between the two disciplines. No doubt, joint action will be a vital step for the development of practical and theoretical applications in both areas. For instance, genetic engineering techniques are very useful in delineating an enzyme mechanism through the use of site-directed mutagenesis. Furthermore, DNA sequencing is almost an indispensable tool for determining the amino acid sequence of a protein. On the other hand, enzymologists can stabilize and improve the use of enzymes needed for DNA manipulations. Perhaps, they might even design artificial restriction enzymes (Dervan, 1986).

2. IMMOBILIZATION OF ENZYMES

Immobilization of enzymes and other biomolecules is a widespread and well known technique in biochemistry (for review see Scouten, 1983; Mosbach, 1976 and Mosbach 1986). For instance, immobilized enzymes have been used for synthesis of useful compounds, in biosensors, such as enzyme electrodes and enzyme thermistors, and for therapeutical applications for removal of toxic substances from body fluids. Immobilization of enzymes also offers an attractive model for studying the protein under the conditions found *in vivo*. Thus, immobilization has been used to study enzyme sequences and subunit interactions in oligomeric enzymes.

Also in the area of recombinant-DNA technology interest has been devoted to immobilization of the enzymes employed in DNA manipulations. This is largely due to their relatively low stability and high cost.

2.1. IMMOBILIZATION PROCEDURES

A large variety of materials has been used as immobilization supports, mostly solid phase beads (e.g. agarose, acrylate and glass) but also membranes, fibers and in few cases, water-soluble supports such as dextran. Enzymes are usually immobilized on these matrices by one of the following methods:

- (a) covalent attachment
- (b) entrapment within a gel lattice or microencapsulation within semipermeable membranes
- (c) adsorption, including hydrophobic and affinity binding
- (d) cross-linking

All methods except the last one have been used for immobilization of enzymes used in DNA manipulations (TABLE 1).

In all cases the immobilized preparation has been reported to catalyze the same reaction as the enzyme free in suspension. Thus, immobilization *per se* does not appear to interfere with the normal catalytic properties associated with the soluble enzymes.

2.1.1. Choice of immobilization procedure

Entrapment and adsorption is usually less common because of possible leakage. However, initial

TABLE 1. Immobilization of enzymes used in DNA manipulations.

Enzyme	Method	Reference
<u>Bam</u> HI	CnBr Tresyl chloride Trityl agarose (adsorption)	Lee <i>et al.</i> , 1978 Bülow, unpublished data Cashion <i>et al.</i> , 1982
<u>Eco</u> RI	CnBr Tresyl chloride Trityl agarose Agarose entrapment	Lee <i>et al.</i> , 1978 Bülow, unpublished data Cashion <i>et al.</i> , 1982 Singh, 1984
<u>Hind</u> III	Trityl agarose	Cashion <i>et al.</i> , 1982
<u>Pst</u> I	CnBr Agarose entrapment Polyacrylamide entrapment	Singh, 1984 Singh, 1984 Singh, 1984
T4 DNA ligase	Tresyl chloride CnBr	Paper I Paper I
T4 RNA ligase	Trityl agarose	Cashion <i>et al.</i> , 1982
Alkaline phosphatase	Tresyl chloride Trityl agarose	Bülow, unpublished data Cashion <i>et al.</i> , 1982
DNA polymerase	Trityl agarose	Cashion <i>et al.</i> , 1982
AMV reverse transcriptase	Trityl agarose	Cashion <i>et al.</i> , 1982
RNA polymerase	Trityl agarose	Cashion <i>et al.</i> , 1982
S1 nuclease	Trityl agarose	Cashion <i>et al.</i> , 1982
Polynucleotide kinase	Trityl agarose	Cashion <i>et al.</i> , 1982

recovered enzyme activities of these procedures often approach 100 %. In the case of entrapment, polyacrylamide is probably the most frequently used support. A disadvantage of polyacrylamide entrapment is that the enzymes can be inactivated by the heat liberated during polymerization. For instance, the heat labile restriction enzyme *Pst* I could not be entrapped in this matrix (Singh, 1984). Normally, long-term stability is inadequate using these methods which can be explained either by protein loss or protein denaturation.

Therefore, major emphasis should be put on covalent attachment. The most commonly used procedure for coupling enzymes to agarose gels, involves activation by cyanogen bromide (March *et al.*, 1974). However, the linkages formed are known to be labile and the tresyl chloride method therefore offers an attractive alternative (Nilsson and Mosbach, 1981). Immobilization through this latter method usually gives rise to enzyme preparations with excellent long term stability, which can be illustrated by immobilized T4 DNA ligase (Figure 2).

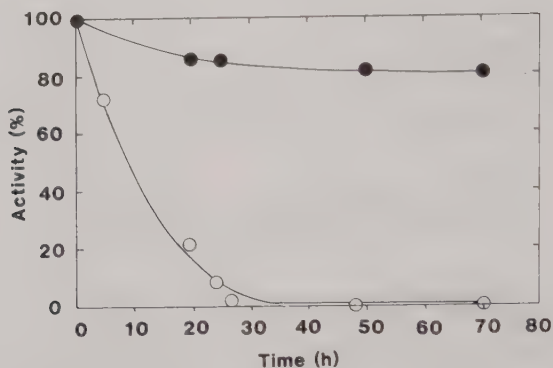


Figure 2. Stability of soluble and immobilized T4 DNA ligase (Paper I).
(●, immobilized enzyme; ○, soluble enzyme)

A major disadvantage with covalent coupling is the potential inactivation of the enzyme caused by modification of essential catalytic groups during immobilization. The recovered activities of restriction enzymes and ligases are indeed comparatively low, usually in the range of 5-20 %. This could be explained by the fact that essential lysines, which are the amino acids on the proteins usually employed for coupling, are involved in general electrostatic interactions with phosphates in the DNA backbone (Woodhead and Malcolm, 1980). Therefore, the degree of activation of the matrix must be carefully checked (Paper I, Olszewski and Wasserman, 1986). Immobilization in the presence of DNA is an approach which might solve the problem.

2.2. APPLICATIONS

An immobilized enzyme has the following advantages in relation to a comparable enzyme free in suspension:

- (a) It can easily be separated from the nucleic acids and tedious phenol extractions are avoided. Thus, when coupled to a beaded polymer, e.g. Sepharose, the enzyme can be rapidly pelleted by centrifugation.
- (b) Its thermal stability is usually increased.
- (c) It can be reused.

However, on the same time it suffers from the following disadvantages:

- (a) A reaction mixture containing nucleic acids is viscous, implying that the rate of diffusion of substrate into and product out of the active site may be reduced when the enzyme is immobilized.
- (b) The enzymic activity can be adversely affected by the chemical reaction involved in immobilization.

If these features are taken together, it can be envisaged that the major field of application of immobilized enzymes will probably be in the area of preparative scale *in vitro* recombination of DNA molecules. Thus, immobilized enzyme columns can be used for large scale cleaving and ligating of DNA, allowing treatment of large amounts of DNA with few units of enzymes. Immobilized T4 RNA ligase has thus been used for the synthesis of nucleic acids with defined sequences (Tan and Hu, 1981) and oligonucleotides prepared by chemical means can be ligated on a semi-preparative scale using immobilized T4 DNA ligase (Paper I, Bülow, unpublished data).

However, also for analytical work immobilized enzymes are of interest. A partial digest with a restriction enzyme is preferably made with an immobilized enzyme preparation. Moreover, dephosphorylation of nucleic acids with alkaline phosphatase should be made with an immobilized enzyme since complete removal of this protein from the nucleic acid is exceedingly difficult.

Besides the practical applications described, immobilization offers a valuable tool for mechanistic studies of these enzymes. For instance, restriction enzymes are potentially membrane bound (Smith *et al.*, 1976), and immobilization should give a clue to which effect this fact might have on their activity. It has been suggested that T4 DNA ligase acts both in the monomeric as well as in the oligomeric state catalyzing ligation of sticky and blunted ends, respectively. By studying the immobilization of monomers and oligomers, respectively, valuable conclusions can be drawn from the kind of activity observed in each case.

3. IMMOBILIZATION OF GENETICALLY ENGINEERED BACTERIA

The use of cell immobilization offers a valuable alternative compared to the conventional fermentation techniques using cells in suspension. Immobilized microbial cells have thus currently found industrial application in continuous production of a variety of useful compounds such as aspartic acid, 6-amino penicillanic acid, malic acid, high fructose syrup and ethanol (Mosbach, 1986). Hitherto, genetically engineered bacteria have been batch-fermented to produce the desired protein. However, the commercial exploitation of recombinant-DNA technology will require the long-term culture of these strains and since immobilized cells have their greatest value in continuous processes this technique will probably be very useful for large scale production of cloned gene products (Paper II; Inloes, *et al.*, 1983; Dhulster *et al.*, 1984). The benefits of using immobilization comprise:

- (a) Immobilized cells are concentrated to **higher densities** within the support material than is possible in normal suspension cultures. Thus, product formation per reactor volume is often higher with immobilized cells. This can be illustrated by *E. coli* W3110i^q(K8) producing human proinsulin, which was immobilized at various concentrations (Figure 3). In the immobilized state this strain can produce at least 4.3 μg proinsulin per ml growth medium while free in suspension it specifies between 1.1 and 1.5 μg proinsulin/ml (Bülow and Birnbaum, unpublished data).

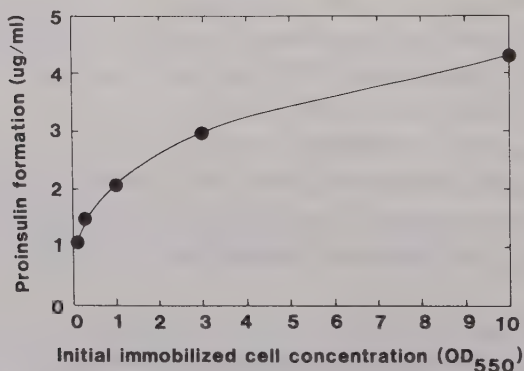


Figure 3. Formation of proinsulin by *E. coli* W3110i^q(K8) immobilized at various concentrations.

- (b) Unlike the chemostat, fermentor wash-out is not a problem at high substrate flow rates. Thus, the process can work **independently of cell growth**. This is an important feature because culture reversion can be avoided by inhibiting cell division and thereby retaining the culture's original genetic specificity.
Additionally, metabolic products can easily be removed before accumulating to inhibitory levels.
- (c) Frequently, there is an **increase in cell stability**. There might also be a physiological change caused by immobilization as indicated, for instance by improved plasmid inheritability (de Taxis du Poët *et al.*, 1986).
- (d) The **separation** of the substrate/product from the cells **is simple**. The catalyst is easy to retrieve and reuse in batch processes as well as to pack into columns.

3.1. IMMOBILIZATION PROCEDURES

In principle, the association of cells with a support can be accomplished by the same procedures as outlined for immobilized enzymes (Chapter 2.1.). Entrapment is generally a gentle and simple method. It allows for high cell densities and is therefore often preferred when immobilizing cells. This approach is also advantageous in comparison with other immobilization methods when cell growth occurs since the majority of daughter cells will remain entrapped within the gel matrix.

The use of mild immobilization procedures, such as entrapment in polysaccharide gels, is essential in order to retain the metabolic and physiological capacities of the immobilized preparation. Furthermore, it is important to use such methods that allow testing of the metabolic activity of the entrapped cells. Formation of a cloned gene product is such an easily monitorable method of assuring cell viability. In paper II this method was complemented with oxygen uptake measurements (Somerville *et al.*, 1977). Otherwise, an efficient test of cell viability involves the dissolution of the support followed by plate spreading and colony counting.

3.2. SUPPORT MATERIALS

A wide variety of support materials have been used for cell immobilization (Birnbaum *et al.*, 1983, Mosbach 1986). Good mechanical stability and diffusibility are essential demands which must be put on the support material. The use of agarose entrapment has proved very useful :

- (a) It has **big pores**, as indicated by the rapid release of ^{125}I -labelled insulin (Figure 4).
- (b) It is **non-toxic**. As opposed to the usually employed polyacrylamide entrapment method, it does not require organic solvents.
- (c) **Small beads** can be formed (Nilsson *et al.*, 1983).

However, for large scale production a cheaper support material must be used.

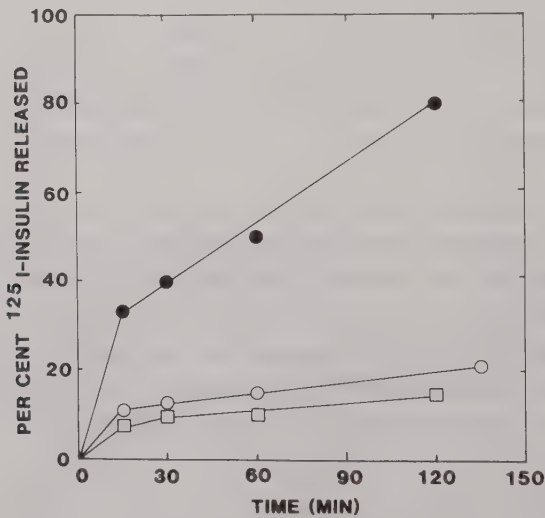


Figure 4. Release of ^{125}I -insulin from different matrices. ^{125}I -insulin was entrapped in 2 % (w/v) agarose (—●—), 2 % (w/v) alginate (—○—) and polyacrylamide, 10 % (w/v) acrylamide and 0.5 % (w/v) methylene bisacrylamide (—□—). The formed beads (diameter approximately 2 mm) were incubated in 0.1 M Tris-HCl pH 7.5 and the ratio of released radioactivity to the buffer to total entrapped was determined.

3.3. HOST CELLS AND PROTEIN SECRETION

When genetically engineered bacteria are immobilized the cloned gene product should be extracellular. The host of choice in recombinant-DNA technology is *E. coli* since a great deal is known of its inherent properties. However, in *E. coli* secretion proteins are normally only transported to the periplasmic space and not to the growth medium. Thus, the use of Gram-positive bacteria such as *Bacillus* species, especially *B. subtilis*, rather than *E. coli* is preferable.

Most secretory proteins are synthesized initially as preproteins which have additional amino acids, the signal sequence, at their N-termini. A protein which is normally secreted by a eukaryote, for instance a hormone, can also be made to be secreted in bacteria. However, *Bacillus* signal sequences differ from those of Gram-negative bacteria and eukaryotes in being longer, which implies that the *Bacillus* signal should be used when a gene coding for an export protein is expressed in these bacteria (Doi *et al.*, 1986). In paper II a hybrid signal sequence comprising the first 15 amino acids of *Bacillus licheniformis* penicillinase signal followed by the signal sequence of rat preproinsulin was employed. Such a hybrid signal allowed for an efficient transport to the extracellular medium.

Recently, *E. coli* mutants having a defective outer membrane, periplasmic leaky mutants, was described (Lazzaroni and Portulier, 1981). Such strains are a valuable alternative to Gram-positive hosts.

Another approach involves the immobilization of *E. coli* minicells. Minicells are small anucleate cells which are produced continuously during growth of certain mutant strains. Minicells produced from plasmid carrying parents contain significant amounts of plasmid DNA. Plasmid containing minicells are capable of RNA and protein synthesis. Such immobilized microbial systems might be very useful for immobilization since there are no demands for cell growth on protein formation (see Chapter 3.4.). So far immobilized minicells have been used only for enzymic bioconversions (Khachatourians *et al.*, 1982), but protein secretion should be feasible also for this system.

3.4. CONTROLLING CELL GROWTH

Formation of polypeptides by bacteria requires that transcriptional and translational machineries of the cell are functional. Normally, product formation is closely associated with cellular proliferation. Such cell division often causes problems in fermentation processes based on immobilized preparations as the cells often clog the matrices in which they are embedded, thus impeding the flow of nutrients and eventually stopping the process. Additionally, because of cell division, the

cells become dislodged from the support and contaminate the product.

Methods for controlling cell growth frequently focus on limitations of nutrients, usually of the carbon or nitrogen source. However, during preliminary studies with the described *E. coli* and *B. subtilis* strains, it was found that growth inhibition using such methods concomitantly led to reduced formation of insulin.

Another approach involving surface sterilization of immobilized *B. subtilis* producing alfa-amylase was described by Kokubu *et al.* (1978). Such sterilization was achieved by immersing the immobilized preparation in 70 % ethanol intermittently after the production phases.

A procedure where DNA synthesis and thus cell growth is inhibited specifically without affecting RNA and protein synthesis is described in paper II. Addition of certain antibiotics, such as nalidixic acid and novobiocin, has proved useful in this respect. These antibiotics act by inhibiting supercoiling of DNA catalyzed by DNA gyrase and they thereby inhibit DNA replication (Gellert *et al.*, 1976). DNA replication can also be inhibited through inhibiting DNA polymerase. Thus, 6-(p-hydroxyphenylazo)-uracil, which is a specific inhibitor of *B. subtilis* DNA polymerase (Brown, 1971), has similar effects as the previously described antibiotics.

The amounts of antibiotics as well as the time of addition must be chosen with care for each recipient strain. TABLE 2 gives a guideline (Bülow *et al.*, 1986) to appropriate additions.

TABLE 2. Concentrations of antibiotics ($\mu\text{g/ml}$) suitable for inhibiting cell division while allowing protein synthesis.

	nalidixic acid	novobiocin	6-(hydroxyphenylazo)-uracil
<i>B. subtilis</i>	20	5	10
<i>E. coli</i>	5	25	

A critical question is the long-term effect of these antibiotics on bacteria. In the case of *B. subtilis* formation of proinsulin can continue between four and seven days. Eventually, the DNA synthesis inhibitors will be lethal to the cells. However, preliminary investigations have shown that the cells can be recovered if the inhibitor is removed, after which the growth inhibition can continue. This

cycle can be repeated about three times. Furthermore, a method for removing the antibiotic from the growth medium must be developed due to the potential risk of contamination of the final product.

An interesting additional feature is that novobiocin stimulates the synthesis of proinsulin approximately two-fold in *E. coli* (Birnbaum and Bülow, unpublished observation).

A similar approach to the use of DNA replication inhibitors involves the use of bacterial mutants which are blocked in DNA replication at elevated temperatures. This inhibition which obliterates the need of antibiotic addition, is reversible on transferring the culture back to the permissive growth temperature. Temperature sensitive mutants can continue protein synthesis for several hours but these mutants are still less characterized (Veomett and Kuempel, 1973).

3.5. OXYGEN SUPPLY

Most bacteria have a high demand for oxygen whilst growing. *B. subtilis* the most frequently used *Bacillus* species in gene cloning, is obligate aerobe while *E. coli* is facultative anaerobe. Oxygen supply is often a limiting factor for actively metabolizing immobilized cells. Therefore, the cells usually form a layer near the gel surface, limited to the outer 50 μm of the gel beads, due to the limited diffusion of especially oxygen, but also other nutrients, into the interior of the beads. Thus, the use of small beads improves the oxygen mass transfer. Furthermore, choice of reactor is important. Continuous stirred tank reactors and air bubble columns (de Taxis du Poët *et al.*, 1986) are two reactors that will assure for efficient oxygen supply. Improvements may also be obtained by using alternative host cells, for instance *B. circulans* which is facultative anaerobe instead of *B. subtilis*.

Several ingenious approaches have been developed to further improve oxygen supply. Addition of hydrogen peroxide to the growth medium in combination with immobilized cells containing co-entrapped manganese dioxide (Brodellus *et al.*, 1981), catalase (Holst *et al.*, 1982) or active carbon (Szwajcer *et al.*, 1982), any of which will convert the peroxide to the desired oxygen. However, hydrogen peroxide can be deleterious to the cells. Recent methods using co-immobilization with algae cells that photolytically will generate oxygen appear to be more attractive (Wikström *et al.*, 1982 and Adlercreutz *et al.*, 1982). Alternatively, the addition of oxygen carriers, such as perfluoro compounds (Adlercreutz and Mattiasson, 1982) or silicon emulsions (Leonhardt *et al.*, 1985), to the growth medium can be used to increase the oxygen concentration.

4. BIFUNCTIONAL ENZYMES

Bifunctional (or polyfunctional) enzymes are defined as proteins consisting of a single type of polypeptide chain but having two (or more) catalytic or binding functions (Kirschner and Bisswanger, 1976). In this context, the conceptional distinction between the closely related multienzyme complexes and multifunctional proteins should be noted. In multienzyme complexes the various activities are contributed by different subunits that associate with each other by noncovalent interactions. In this thesis bifunctional enzymes have been studied from two points of view:

1. Bifunctional enzymes in enzyme sequences.
2. Bifunctional enzymes as a tool for the design of enzyme-like catalysts from the "ground up" (Van Brunt, 1986) (Chapter 5).

4.1. BIFUNCTIONAL ENZYMES IN ENZYME SEQUENCES

Several lines of experimental work have proved that the arrangement of enzymes and proteins in relation to one another is essential to cell metabolism (Welch, 1985). This fact can also be realized by a purely logical reasoning. Optimizing cell metabolism can be done by improving the catalytic efficiency of the individual enzymes. However, the protein structure itself cannot increase the rate at which the substrate diffuses to the enzyme. Formation of multienzyme complexes and/or multifunctional enzymes thus provide for coordination and unification of enzyme activities. Alternatively, enzyme sequences can be organized in or on cell membranes.

The naturally occurring bifunctional enzymes are predominantly found in the pathways of amino acid biosynthesis. In the majority of known cases, the separate active sites catalyze sequential metabolic steps: $A \rightarrow B \rightarrow C$.

What kind of selective advantages has a bifunctional enzyme had compared with two separate enzyme activities during evolution? Gaertner (1978) put forward that the organization of enzymes into multienzyme systems may offer the following advantages:

1. **Coordination effects.** An entire sequence of enzymes can be coordinately activated or inhibited. An example of this phenomenon has been found for the *arom* conjugate, a multifunctional protein with five distinct sequential enzymes residing on a dimer of a single

polypeptide chain. Four out of the five enzyme activities on the polypeptide chain are activated by the first substrate. In addition, all five activities appear to be coordinately protected from proteolysis when the first substrate is present.

2. **Compartmentation.** A multienzyme system has the potential of compartmentalizing or containing the substrate of a pathway, which implies that the system prevents interference from an enzyme activity outside the metabolic sequence (channeling).
3. **Elimination of lag phases.** The intermediate substrate formed does not or at least only partially diffuses out into the surrounding medium, thereby can a high local optimal concentration of substrate for the enzyme be obtained.
4. **Reduction of diffusion times.** Diffusion can be the rate limiting step in an enzyme reaction if the surrounding medium is viscous.

To this list should be added that unstable intermediates can be protected. Moreover, the intrinsic catalytic properties of the multienzyme system might be different from the separate proteins.

The covalently linked proteins also present the following additional advantages in relation to the multienzyme complexes:

1. **Biosynthesis is more efficient.** The most effective coordination of synthesis of the involved enzymes is the combination of the genetic loci into a single unit coding for a single polypeptide.
2. **Increased stability.** A noncovalently associated aggregate might disassociate at a high dilution (McCarthy and Hardie, 1984).

The enzymes of different organisms which are involved in tryptophan biosynthesis represents a bewildering variety of gene arrangements as illustrated by the presence of individual proteins, multienzyme complexes and multifunctional enzymes (Miles, 1979). These observations early led to the hypothesis that evolution has proceeded from independent small proteins to multienzyme complexes or multifunctional proteins. Recently, this hypothesis has received strong support when nucleotide sequences of the bifunctional enzymes and the corresponding separate enzymes have become known. A large degree of homology is thus found. This can be illustrated by the tryptophan synthases of *E.coli*, *Neurospora crassa*, *Saccharomyces cerevisiae*.

4.1.1. Tryptophan synthase

Tryptophan synthase catalyzes the synthesis of tryptophan from serine and indoleglycerol phosphate in two consecutive steps:



In *E. coli* and other prokaryotes tryptophan synthase is a tetramer made of two α monomers and one β_2 dimer. Tryptophan synthase from *S. cerevisiae* catalyzes the same partial reactions and has been shown to be a dimer of identical bifunctional polypeptide chains (α/β)₂ (Miles, 1979). The α/β subunit is slightly larger ($M_r = 76,000$) than the sum of the molecular weights of *E. coli* α ($M_r = 29,000$) and β ($M_r = 43,000$) chains (Dettwiler and Kirschner, 1979). The NH₂-terminal segment of yeast tryptophan synthase is homologous with *E. coli* tryptophan α subunit, while the COOH-terminal segment is homologous to the β subunit.

The α and β chains of the *E. coli* enzyme are translational products of a polycistronic mRNA transcribed from the *trpEDCBA* operon while TRP5 is the structural gene of yeast tryptophan synthase (Zalkin and Yanofsky, 1982).

The nucleotide sequence of the connecting region separating the contiguous *trpB* and *trpA* genes consists of overlapping stop and start codons UGAUG (Nichols and Yanofsky, 1979). It is obvious that a single base pair insertion in this punctuation region can fuse the structural genes. However, a bifunctional tryptophan synthase having a B chain/A chain fusion has yet to be found. TRP5 encodes an A-chain/B-chain fusion, thus the order of the genes has been reversed. Moreover, the bifunctional enzyme carries a connecting region between the catalytic domains. This "spacer" may be required for a correct folding and interaction of the catalytic entities. It is plausible that the spacer reflects the DNA sequence associated with the genetic events that led to a fusion. At least a portion of the spacer is homologous to the 3'-end of *E. coli trpC* and the *trpC-trpB* intercistronic region.

Substrate channeling

One of the most thoroughly studied characters of these enzyme "clusters" is the spatial relationship

between the active centers or the possibilities of substrate channeling. Frequently, such investigations are based on the kinetic behaviour of the intermediate. If the sites are juxtaposed, the product of the first reaction can be processed in the second step as it becomes available and does not accumulate in the medium. If the active centers do not interact (overlap) the intermediate must find the second site by free diffusion and formation of the final product proceeds with a distinct lag. An active site arrangement for the *E.coli* enzyme has been proposed based on the observation that no exchange took place between the solvent indole surrounding the protein and the indole moiety of indole glycerol phosphate. Therefore, it seems likely that the indole from indole glycerol phosphate remains confined within a very limited space during the catalytic process (Creighton, 1970). Also in the case of the *Neurospora crassa* enzyme, a bifunctional enzyme, it was shown that the transfer of the intermediate, indole, from the first to the second site was about 200-fold more probable than its escape to the solvent (Matchett, 1974).

However, the two active sites of the *E.coli* enzyme might not be in close physical contact with each other. Attempts to chemically cross-link the active sites using short bifunctional reagents have not been successful, indicating that the active sites might not be juxtaposed but rather that channeling of indole is caused by a hydrophobic region (Heilman and Holzner, 1981).

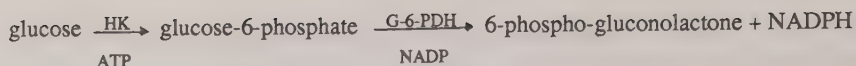
4.2. MODEL SYSTEMS

The spatial organization of enzymes appears to be the rule rather than the exception in reflecting enzyme sequences *in vivo*. Therefore, when designing model systems for metabolic studies, the arrangement of proteins must be taken into consideration. Different approaches have been employed to create proximity between the enzymes. Thus, sequentially operating enzymes can be:

- (a) Co-immobilized to a solid matrix.
- (b) Chemically cross-linked in a random or an oriented fashion.
- (c) Ligated by fusion of the structural genes.

4.2.1. Co-immobilization of sequentially operating enzymes

Mosbach and Mattiasson (1970) co-immobilized a two enzyme system comprising hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH) which catalyzes, the sequential reactions:



The initial rate of formation of the final product NADPH (or 6-phospho-gluconolactone) was considerably enhanced when the two enzymes were co-immobilized, as compared with when they were soluble or immobilized on separate beads. A combination of two factors, the proximity of the enzymes and the hampered out-diffusion of intermediates due to the unstirred layer surrounding the beads, was proposed to explain this effect. Theoretical calculations by Goldman and Katchalski (1971) have confirmed this kinetic behaviour of two-enzyme systems.

Several immobilization experiments with other enzyme sequences and cyclic enzyme systems have since then been reported, all establishing the favourable kinetic effects of co-immobilized enzyme systems (Welch, 1985).

4.2.2. Chemical cross-linking

In an attempt to evaluate the contribution of proximity versus diffusion hindrance Mattiasson *et al.* (1974) and Koch-Schmidt *et al.* (1977) chemically cross-linked two sequentially acting enzymes. Enzyme conjugates of malate dehydrogenase and citrate synthetase was thus prepared by glutaraldehyde cross-linking. In an ordinary buffer solution this bis-enzyme was found not to be more efficient than a reference system comprised of non cross-linked enzymes. However, a factor of great importance to the efficiency of an enzyme sequence is the concentration of other proteins in the vicinity of the studied enzymes. The high protein concentrations found *in vivo* can lead to a partial structuring of water in the microenvironment of the enzymes. The presence of proteins would thus cause a reduction in volume of non-associated water available for solute molecules, e.g. the substrate, surrounding the enzymes. Addition of polymers, like poly(ethylene glycol) (PEG) to the assay mixture causes similar exclusion effects as high protein concentrations. Such PEG addition resulted in a kinetic advantage, in the form of increased steady-state rate, of the cross-linked enzymes over the corresponding free enzymes.

In naturally occurring enzyme sequences the constituent enzymes are in many cases probably not randomly associated. A more sophisticated model is therefore the preparation of chemically cross-linked oriented enzyme aggregates, such as the alcohol dehydrogenase-lactate dehydrogenase system described by Månsson *et al.* (1983). Close juxtaposition of the active site was achieved by orienting the active sites with bis-NAD prior to chemical cross-linking. This bis-enzyme complex provided a suitable microenvironment for cycling of the intermediate NAD(H) as tested by using a

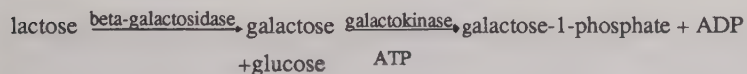
competitive enzyme, lipoamid dehydrogenase.

4.2.3. Gene fusion

A bifunctional enzyme can be prepared by ligating the structural genes of two enzymes. The translational stop signal at the 3'-end of the first gene and the promoter region at the 5'-end of the second gene are removed and upon fusion the novel gene thereby encodes a single polypeptide chain carrying both active sites. A simple example of creating such a bifunctional enzyme would be to remove the punctuation signals in an intercistronic region. Youmo *et al.* (1970) achieved this by "classical" mutations and managed to fuse the second and third genes (*hisD* and *hisC*) of the histidine operon of *Salmonella typhimurium*. With the development of genetic engineering techniques there are no restrictions to fuse only adjacent genes but also chromosomally distant genes can be ligated.

A lot of structural genes have been employed for fusion purposes, e.g. beta-galactosidase (*lacZ*) (Casadaban *et al.*, 1983), galactokinase (*galK*) (Rymond *et al.*, 1983), alkaline phosphatase (*phoA*) (Matteucci and Lipetsky, 1986) and beta-lactamase (*bla*) (Villa-Komaroff *et al.*, 1978).

Two of these genes, *lacZ*, and *galK*, were ligated and expressed in *E.coli*. The obtained artificial bifunctional enzyme, beta-galactosidase/galactokinase is able to catalyze the normal sequential reactions:



This fact indicates that the hybrid protein is capable to form the functional domains associated with the wild-type enzymes.

Physical properties

The hybrid protein has a molecular weight corresponding to the sum of the wild-type enzymes. Beta-galactosidase is a tetrameric enzyme while galactokinase is a monomeric enzyme. The configuration of beta-galactosidase/galactokinase is tetrameric but also larger aggregates can be detected, with each beta-galactosidase monomer carrying a galactokinase unit (Figure 5).

However, the tertiary structure of the hybrid protein is most likely somewhat disturbed or put under

some "physical stress" as indicated by a lower thermostability. By employing immunological methods Fowler and Zabin (1983) discovered that beta-galactosidase, in the fused state, appears to have a more "open" configuration than the wild-type enzyme.

The close proximity between the proteins creates a new microenvironment which in turn affects the catalytic properties of the individual enzyme activities. For instance, the pH activity profiles of beta-galactosidase and the K_m value for the charged substrate, ATP, are changed. This can be explained by novel, charged surroundings created in the gene fusion event.

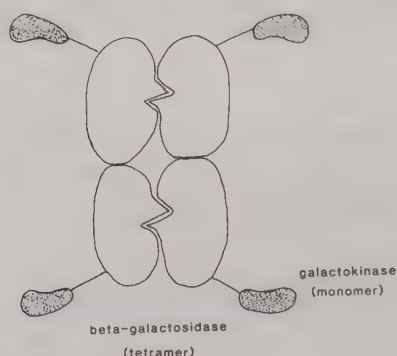


Figure 5. Schematic configuration of the beta-galactosidase/galactokinase enzyme complex.

The role of substrate channeling

When studied alone the bifunctional enzyme appears to have no kinetic advantages over a comparable system with wild-type enzymes. For instance, no differences in the lag phase or steady-state rates could be detected. However, when a competing enzyme, galactose dehydrogenase was introduced at least partial substrate channeling was discovered (Figure 6). The effect became even more pronounced in assay media containing increasing concentrations of poly(ethylene glycol).

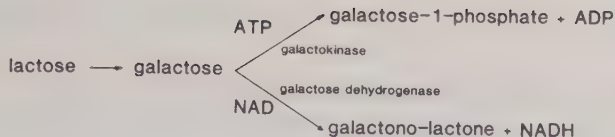


Figure 6. Schematic representation of the three enzyme system used for the "scavenger" enzyme assay.

What are the grounds of substrate channeling?

Part of the effect can most likely be found in the fact that the distance between beta-galactosidase and galactokinase is much shorter than between beta-galactosidase and galactose dehydrogenase even though a juxtaposition of the active sites of the hybrid protein appears less probable. However, differences in diffusion times between the bifunctional and the wild type enzymes cannot be the sole explanation. The possibilities of "surface diffusion" of the intermediate must be evaluated. This automatically brings up the question of the thickness of the "unstirred layer" around globular proteins. Theoretical calculations by Hagler and Moulton (1978) have suggested such a thickness of lysozyme of no more than one monolayer of water molecules, but theoretical and practical investigations of other proteins are still lacking.

Connecting region

All of the naturally occurring multifunctional proteins that have been sequenced contain a "hinge" region between the catalytic entities. These connecting regions are considered to be remnants of uncestral flanking regions of the structural genes in question. Therefore, when nucleotide sequences of different connecting regions are compared, no homology can be found (Zalkin *et al.*, 1984).

The functional role of these regions are unknown. They might give the complex a conformational flexibility. Two beta-galactosidase/ galactokinase hybrids with and without a "spacer" have been prepared. At least the galactokinase activity is not affected by the spacer. It is difficult to evaluate the beta-galactosidase moiety, since in the case without spacer, COOH-terminal amino acids involved in subunit interactions were also missing. However, variations in this linker regions, e.g. introduction of a steric amino acid sequence like polyproline compared with a flexible one, like polyglycine, should allow a delineation of the relative contribution of active site proximity versus unstirred layer.

4.3. PROTEIN FUSION

A fascinating alternative to the above purely genetic approaches consists of the direct covalent linkage of separately synthesized polypeptide chains. Protein fusion has been detected *in vivo* in a few cases, but the molecular mechanism is still known (Hendrix and Casjens, 1974).

A beta-galactosidase/galactokinase hybrid protein can be prepared by this latter approach. If galactokinase is modified by introducing a polycysteine tail in the NH₂-terminal of the protein, in the absence of thiol reagents, it will interact strongly *in vitro* with beta-galactosidase, known to have at least 12 free SH groups available for coupling (Bülow, unpublished data). Wildtype beta-galactosidase and galactokinase do not interact with each other.

5. ARTIFICIAL ENZYMES

Despite the fact that biochemists have isolated and characterized hundreds of enzymes, the industrial use of enzymes is limited. This often stems from the fact that the required industrial application is far removed from the physiological role normally played by the enzyme. For instance, industrial processes are frequently carried out at elevated temperatures, however, enzymes often denature above 60°C, except enzymes of thermophilic organisms. Furthermore, gas phase reactions and organic solvents, which often are detrimental to enzymes (however, cf. Zaks and Klibanov, 1985), are commonly used in industrial production plants. In addition, there are many chemical conversions of interest for which no enzyme exists or for which an enzyme catalyzes the reaction poorly. If enzymes are to be more widely employed, their properties must be tailored to the process of interest. Today, site-directed mutagenesis and design of novel enzyme reactors are the techniques to choose between. These approaches might help on some occasions, but the design of an entirely new catalyst, an artificial enzyme, will probably be a better resource in the long run.

Unfortunately, it is still impossible to predict the tertiary structure of a large protein from its amino acid sequence alone, which makes it difficult to design an artificial enzyme. However, protein chemists are beginning to learn how to design sequences of amino acids that will fold up into regular repeating structures. Two such folding patterns occur in natural proteins. In the alpha, or helical, pattern the main chain forms a tight coil stabilized by internal hydrogen bonds, with the amino acid side chains extending outward in a helical array. The beta, or pleated-sheet, pattern consists of parallel strands of protein chains stabilized by hydrogen-bonding interactions between the strands.

These two folding patterns are the foundation for the design of artificial proteins from basic structural principles. The area is still in its embryonic state but a few artificial proteins designed from the ground up have been described. A synthetic polypeptide based on the beta sheet structure which binds the insecticide DDT was recently successfully designed and chemically synthesized (Moser *et al.*, 1983). It consists of 24 amino acids which fold up into a four-stranded beta sheet with 6 amino acids in each strand.

Two other prototypical binding proteins, Felix and betabellin, have been designed by molecular graphics and energy minimization techniques (Van Brunt, 1986). Felix consists of a bundle of four connected alpha helices while betabellin, which is an abbreviation of "beta barrel bell-shaped protein", consists of two identical chains of 31 amino acids, which fold up into a pair of beta

pleated sheets with hydrophobic amino acids on one side and hydrophilic amino acids on the other.

However, polypeptides are large molecules. Therefore, the assembly of ingenious, much smaller molecules not based on a polypeptide backbone, so called synzymes, offer an attractive alternative as carriers of enzyme activity. Molecules such as cyclodextrin (Breslow, 1982) and cavitands (Cram, 1983) can be tailored to share some of the binding and catalytic properties of their larger counterparts, the enzymes.

Which of the two approaches are preferable?

With present-day technology synzymes often require complex organic syntheses, precluding widespread use. A polypeptide chain is easily synthesized, but the folding and catalytic properties are difficult to predict. Taken together, the methods are probably equivalent and the development of both approaches should preferably run parallel with each other. Thus, the synzymes and enzymes are competing for the same market. This fact can be illustrated by the numerous designs of enzyme-like esterases published which all mimic serine proteases and where both peptides and synzymes have been prepared.

5.1. SERINE PROTEASE MIMICS

Serine proteases are characterized by the presence of a serine, a histidine and an aspartic acid in the active center. In the first step of hydrolysis of esters, the serine group is acylated, and the alcohol part of the substrate is displaced. Hydrolysis of the acyl serine intermediate completes the reaction. It should be noted that when this reaction is characterized only the first step, the acylation, has usually been taken account for. Thus, no true turnover is normally studied.

5.1.1. Synzymes

Cyclodextrins

The cyclodextrins have excited much interest as possible components of artificial enzymes. These components, also called cycloamylases, are naturally occurring cyclic oligosaccharides consisting of 6, 7 or 8 glucose residues in α -1,4 linkages. Cyclodextrins are soluble in water but the cavities themselves are hydrophobic. Thus, the cyclodextrins have the ability to extract small organic molecules out of water solution and bind them to the cavities. Cyclodextrin binding is selective for molecules that have the correct shape and hydrophobic character. Catalytic groups can be mounted on the cyclodextrin framework, thereby mimicing enzymes in having both a catalytic and a binding domain.

A chymotrypsin analogue was thus recently prepared by attaching an imidazolyl group, a carboxylate group and a hydroxyl group to cyclodextrin (D'Souza *et al.*, 1985).

Cavitands

Another type of binding sites are the "cavitands", which are synthetic organic compounds containing a cavity suitable for binding. Cram and Katz (1983) have described the synthesis of a macrocyclic compound carrying catalytic groups of the charge relay system of serine proteases (Sigler *et al.*, 1968), which were arranged in close proximity of the binding site.

Polymers

Most enzymes are globular proteins with compact structures. Therefore, it appears that a good enzyme mimic should be based on polymers. Experiments, especially with branched polymers, have yielded encouraging results. A branched polymer that has been studied extensively in this respect is poly(ethylene imine), PEI. Substitutions of the PEI nitrogens with lauroyl and imidazolymethyl groups result in a catalyst capable of ester hydrolysis. Since the hydrophobic lauroyl groups are in the vicinity of the imidazolyl groups on the surface of the polymer, preference for hydrophobic substrates are obtained (Klotz *et al.*, 1971).

5.1.2. Peptides

A number of synthetic oligo- and polypeptides mimicing the active sites of serine proteases have been described, all prepared by conventional organic chemistry. They contain either imidazolyl groups alone or imidazolyl groups in conjunction with carboxyl and hydroxyl groups. In some cases have the hydroxyl groups been substituted to thiol groups. For instance, Cruickshank and Sheehan (1964) synthesized the oligopeptide, thr-ala-ser-his-asp, and Petz and Schneider (1976) his-ala-asp-gly-cys. A number of different polypeptides have also been analyzed for catalytic activity. Thus, Merrifield and Woolley (1957) as well as Katchalski *et al.* (1960) prepared: poly-L-histidine, poly(L-histidyl-L-glutamic acid), poly(L-histidyl-L-seryl-L-glutamic acid). In paper VI a polymer based on the catalytic unit, glu-ala-his-ala-ser-phe-phe-phe, was prepared by genetic engineering methods instead of previously used chemical methods.

5.2. CATALYTIC EFFICIENCY

The catalytic efficiency of the catalysts, both synzymes and peptides, are assayed for by

determining the hydrolytic activities against various activated esters, such as p-nitrophenyl esters of aliphatic carboxylic acids. It is difficult to give an unambiguous comparison between the two groups, since different substrates are used due to different substrate selectivities. Therefore, TABLE 3 only gives a rough estimate of the relative effectiveness of the catalysts in hydrolysis of nitrophenyl esters.

The catalytic activity of all catalysts strongly indicate that imidazole-type catalysis cannot be the sole explanation for the observed rate enhancement phenomena, but rather that some of the polyfunctional effects, i.e. more than one amino acid residue is involved in catalysis, associated with enzyme active sites might indeed be operative in these models.

5.2.1. Substrate specificity

Much attention has been focused on the rate increases caused by the catalysts. However, substrate specificity is probably of even greater importance when designing artificial enzymes.

In paper VI the phe-phe-phe groups of the catalytic esterase units was introduced to promote binding of hydrophobic substrates. Indeed, increased catalytic activity was observed to more hydrophobic p-nitrophenyl esters as compared with e.g. the tripeptide ser-his-asp. Similar results have been obtained by co-immobilizing histamine and octylamine to carboxymethyl Sephadex (Nilsson and Mosbach, 1979).

Cyclodextrin and cavitand based synzymes also have a suitable substrate binding site for organic components but they have a very limited flexibility in substrate specificity.

Another important feature of the catalyst is the stereoselectivity. Lehn and Sirlin (1978) reported on enantiometric preference by a synthetic cyclic organic compound (Figure 7) in thiolysis of dipeptide esters. They found a 50-fold increase of hydrolysis of glycyl-L-phenylalanine p-nitrophenyl ester as compared with the corresponding D-isomer. Also Sheehan *et al.* (1966) found a preference for the L-isomer of N-methoxycarbonylphenylalanine p-nitrophenyl ester using L-threonyl-L-alanyl-L-seryl-L-histidyl-L-aspartic acid as catalyst.

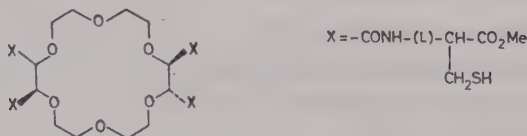


Figure 7. Macrocyclic catalyst exhibiting high chiral recognition for the (L) antipode of glycyl-phenylalanine p-nitrophenyl ester (Lehn and Sirlin, 1978).

TABLE 3. Relative activities of various catalysts in hydrolysis of p-nitrophenyl esters.

(Molar activities are expressed in arbitrary units using histidine·HCl as reference)

Catalyst	Molar activity	Reference
PEPTIDES		
chemically synthesized		
histidine·HCl	1	VI
ser-his-asp	3	VI
thr-ala-ser-his-asp	21	Cruickshank and Sheehan (1964)
his-ala-asp-gly-cys	138	Petz and Schneider (1976)
poly(his-glu)	7,600	Merrifield and Woolley (1957)
genetically engineered		
(glu-ala-his-ala-ser-phe-phe-phe) ₇ -galactokinase	3,200	VI
SYNZYMES		
poly(ethylene imine)- dodecyl -imidazole	620	Klotz <u>et al.</u> (1971)
cyclodextrin-imidazolyl-hydroxyl-carboxyl	5,700	D'Souza <u>et al.</u> (1985)

6. ANALYTICAL APPLICATIONS

Enzymes have found one of their most important fields of application in the area of analysis. This is largely due to their high specificities and frequently high turnover numbers. In this chapter the calorimetric principle of analysis is emphasized. Calorimetry is particularly suitable in biochemical analysis since most enzymic reactions are associated with a considerable heat evolution, frequently more than 20 kJ/mole substrate. The use of calorimetry can be a serious limitation in that all enthalpy changes of the system studied are monitored without discrimination. However, by combining this unspecific method of detection with a specific enzymic reaction such an analytical system will become highly attractive.

One of the most important benefits of calorimetric analysis is the independence of the optical properties of the analytes. Thus, turbid or coloured samples can usually be analyzed without any pretreatment. Furthermore, there is no requirement of colour changes or pH shifts as in spectrophotometry or titrimetry which means that calorimetry offers a greater flexibility in choice of reaction.

Widespread use of calorimetry has yet been hampered by the high cost of equipment and the time-consuming equilibration steps often necessary. The development of simple and comparatively cheap flow calorimeters such as the enzyme thermistor (Mosbach and Danielsson, 1981), which has been used for routine analyses in clinical laboratories and fermentation industries, has improved the situation. The present status of calorimetry in biochemistry must, however, still be regarded as somewhat neglected.

Two areas of application of the enzyme thermistor will be discussed:

- (a) calorimetric detection in enzyme immunoassay
- (b) use of bifunctional enzymes in analysis

6.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Bacterial fermentations are generally characterized and evaluated by monitoring parameters such as pH, pO_2 and pCO_2 . For a more adequate control to achieve optimal conditions a direct determination of the components of interest is preferable. Most proteins produced by genetically engineered cells are quantified by immunological methods. Such uses of immunosorption for

assay of various biological substances in physiological fluids and fermentation broths have received an enormous attention during the last decade. Initially, radioimmunoassay (RIA) was developed (Yalow and Berson, 1959) and has since then been followed by methods based on other markers such as fluorescent probes (Aalberse, 1973), phages (Haimovich *et al.*, 1970) and enzymes (Engvall and Perlmann, 1971). Enzyme-Linked Immunosorbent Assays (ELISAs) are based on the interaction between an antigen and a specific antibody in which one of them is labelled with an enzyme. After complexing to the corresponding matrix-bound antigen or antibody, the non-bound material is separated and an assay for enzymic activity is carried out.

The enzymes used in ELISA can be divided into two groups. The first group comprises horse-radish peroxidase, beta-galactosidase and alkaline phosphatase which are used with separation of the free and bound fractions of the ligand. The second group are those whose activities are altered if an antibody binds to a ligand covalently coupled to the enzyme. These include enzymes like lysozyme, glucose-6-phosphate dehydrogenase and malate dehydrogenase. No separation between the free and bound fraction is required and these assays are thus called homogeneous assays. Usually, the activities are assayed for spectrophotometrically or fluorimetrically and the sensitivities of ELISA are impressive, usually down to 1 ng/ml for most antigens. These methods are, however, time-consuming and the results are frequently obtained after the fermentation is completed. Thus, the requirement of quick analyses is urgent.

6.1.1. Thermometric Enzyme-Linked Immunosorbent Assay (TELISA)

By combining the ELISA technique with a calorimetric detection using the enzyme thermistor, a much wider choice of enzymes suitable for labelling becomes available, since heat is evolved in most enzymic reactions. This thermometric assay method has been coined TELISA (Thermometric Enzyme-Linked Immunosorbent Assay) (Mattiasson *et al.*, 1977). The enzyme thermistor has been employed mainly in a competitive ELISA. The assay principle is simple. The column, through which a continuous flow is pumped, is filled with antibodies immobilized on Sepharose CL-4B. Before the antigen-containing sample to be analyzed is introduced, a fixed amount of enzyme-labelled antigen is added. On passage of the sample through the immunosorbent, competitive binding of the native and enzyme-labelled antigen will take place. The amount of enzyme bound is measured by registering the heat evolved during introducing a pulse of substrate. After the heat pulse is measured, the system is washed with 0.2 M glycine-HCl, pH 2.2, to split the complex. This dissociation step is critical, since any remaining enzyme activity on the column would severely influence the results of subsequent analyses. Finally, a phosphate buffer is introduced, and the system is ready for another assay.

The TELISA technique has earlier been applied to analyses of human serum albumin (Mattiasson *et al.*, 1977), insulin (Mattiasson and Borrebaeck, 1978) and gentamicin (Mattiasson *et al.*, 1978). In paper III, human proinsulin was analyzed in bacterial fermentation broth. The loss in sensitivity, 0.1 $\mu\text{g/ml}$ for TELISA compared with 1 ng/ml for conventional ELISA, in such a non-equilibrium assay is outweighed by a quicker analysis. The results can be obtained after approximately 7 minutes and the total assay cycle takes about 13 minutes to complete (Birnbaum *et al.*, 1986). Moreover, the samples are assayed sequentially rather than simultaneously giving a more suitable analytical approach in controlling e.g. a bacterial fermentation. The immobilized antibody column can be used over a period of 2-3 weeks allowing about 100 analyses.

6.2. PREPARATION OF BIFUNCTIONAL ENZYME CONJUGATES SUITABLE FOR ELISA BY GENE FUSION

A wide variety of ELISA variants has been developed. All of them have in common the requirement of covalent coupling of an enzyme to the antigen or antibody by chemical means. Chemical cross-linking and the following purification step can occasionally be cumbersome to undertake which renders these essential reagents relatively expensive. However, such reagents can be prepared through fusion of the structural genes in question.

With present-day technology, the preparation of four alternative enzyme conjugates by gene fusion appears feasible:

- (a) antigen/enzyme conjugates
- (b) antigen/part of enzyme conjugates
- (c) antibody/enzyme conjugates
- (d) protein A/enzyme conjugates

As marker enzyme is beta-galactosidase (*E. coli*) the first choice, since many fusion vectors are available (Casadaban *et al.*, 1983). An attractive alternative, however, is alkaline phosphatase of *E. coli* (*phoA*) which is much smaller than beta-galactosidase, $M_r(\text{alkaline phosphatase}) = 80,000$ while $M_r(\text{beta-galactosidase}) = 464,000$. Moreover, alkaline phosphatase is a periplasmic enzyme which should simplify protein purification (Matteucci and Lipetsky, 1986).

6.2.1. Antigen/enzyme conjugates

The simplest example of preparing a marker reagent suitable for ELISA is the fusion of the structural gene of a peptide antigen to an enzyme gene, e.g. *phoA* or *lacZ*. Thus, we are currently preparing fusion proteins between human proinsulin and alkaline phosphatase in *E. coli* which are secreted to the periplasm (Lindbladh *et al.*, 1986, to be published; Peterhans *et al.*, 1986, submitted).

6.2.2. Antigen/part of enzyme conjugates

ELISA can be based on antigen-enzyme fusions where only a part of the enzyme (inactive) is used, which is rendered active upon complementation with an appropriate peptide fragment. This technique is an attractive alternative when the enzyme moiety has become inactive in the gene fusion event. The most well-known example is the beta-galactosidase alfa-complementation (Langley *et al.*, 1975). The antigen peptide can be fused to the C-terminus of truncated beta-galactosidase which when combined with the M15 mutant protein, the alfa-acceptor, forms an enzymatically active oligomer. Based on these principles Miller and Herschberger (1984) designed an alfa-complementation assay for human insulin.

6.2.3. Antibody/enzyme conjugates

A futuristic alternative is represented by antibody-enzyme fusions. Recent techniques, however, allow the stable integration of immunoglobulin gene DNA into myeloma cells which in turn make it feasible to prepare antibodies possessing novel properties. Chimaeric proteins in which the antigen binding portion of the immunoglobulin is fused in frame with an enzyme is of great practical interest. Neuberger *et al.* (1984) thus fused the structural gene of nuclease from *Staphylococcus aureus*, a secreted enzyme, with a mouse immunoglobulin μ gene. This enzyme conjugate proved to be useful for ELISA purposes employing M13 single stranded DNA and ethidium bromide as substrates.

Although nuclease is not the most suitable candidate as a marker enzyme and although the cloning of immunoglobulin genes is still in its infancy, one can already state that such fusions will be very attractive for ELISA.

6.2.4. Protein A/enzyme conjugates

Protein A binds specifically to the Fc part of immunoglobulins of many species and has therefore frequently been employed in immunological assays. Protein A is then marked either with an enzyme or a radioactive isotope. Plasmid vectors, containing the gene coding for staphylococcal protein A, which have been adapted for gene fusion, have been constructed by Nilsson *et al.* (1985). These vectors allow the fusion with any gene and a hybrid fusion protein between an active enzyme and the Fc binding region can thus easily be prepared.

6.3. USE OF BIFUNCTIONAL SEQUENTIALLY ACTING ENZYMES IN ANALYSIS

Bifunctional enzymes offer not only a valuable model for studying cell metabolism as discussed in Chapter 4, but there are many enzymic conversions of practical interest involving the action of sequential enzymes. Previously, Mosbach and Mattiasson (1978) tabulated and reviewed various applied uses of such systems, especially in the form of co-immobilized systems.

Many multistep enzyme systems have been used for analytical purposes. A number of e.g. spectrophotometric analyses are thus based on the sequential action of two or more enzymes to produce an easily monitorable product. Immobilized preparations have frequently proved especially useful in this respect because they allow reuse of these expensive reagents. Furthermore, the enzymes can through immobilization be arranged in close proximity to a measuring device, a transducer.

One such design is the enzyme electrode where the enzymes are confined in a dialysis bag or immobilized into a membrane directly at the electrode surface. For instance, Verduyn *et al.* , (1983) co-immobilized alcohol oxidase and catalase in an electrode system designed to measure alcohol in aqueous solution. Multistep enzyme systems have also been applied to thermal analyses, to amplify the heat response of a primary reaction on a substrate of interest. For instance, co-immobilized glucose oxidase-catalase have proved more efficient in determining glucose than glucose oxidase alone (Danielsson *et al.*, 1977).

To exploit the analytical applicability of the beta-galactosidase/galactokinase hybrid, the bifunctional enzyme was immobilized to controlled pore glass. By pumping a buffer solution with and without ATP through the enzyme thermistor, the contribution of the galactokinase moiety on the heat evolution was evaluated. Under non-optimized conditions approximately a two-fold increase in

lactose sensitivity could be achieved when the phosphorylation step was added to the lactose hydrolysis (Bülow and Mosbach, 1985 and Ljungcrantz *et al.*, 1986, to be published).

7. DISCUSSION AND PROSPECTS

The central topic of this work has been the **formation** of polypeptides in bacteria. This wide-embracing term comprises two closely related terms, **production**, which implies large scale (industrial) use of bacteria, and small scale **synthesis** especially of modified or novel proteins.

Two fundamentally different methods, a biological and a chemical method, are available for protein formation. Therefore, when starting up a protein formation project it is most worthwhile to evaluate the fields of application of each method. In this work has only the biological method been studied, involving cloning and expression of the structural gene in question. In the chemical approach the protein is prepared through conventional organic chemistry. The most commonly used procedure is solid-phase protein synthesis (Merrifield synthesis) which involves growing chains of amino acids on polystyrene beads.

The main advantage of chemical synthesis over genetic engineering techniques is that it provides more freedom for experimentation. While bacteria can make proteins containing only 20 amino acids specified by their genes, the organic chemist has approximately 2,000 natural and man-made amino acids to choose between. In addition, biological systems have evolved to use only one isomer of amino acid, the levo (L), structure. However, chemists can synthesize the mirror-image right-handed, dextro (D), forms and incorporate them into artificial proteins which may have useful properties not found in the (L) forms.

Nevertheless, genetic engineering provides important advantages for large scale production. For larger proteins containing more than 20-30 amino acids, chemical synthesis tends to be costly, has low yields and generates a complex mixture of side reaction products, making the desired protein very difficult to purify.

Thus, for large proteins, such as enzymes, as well as for small proteins (> 50 amino acids) when it is asked for preparative scale, it is on most occasions preferable either to isolate the structural gene or to synthesize the DNA chemically and then clone and express the gene in bacteria. Using a bacterial fermentor, large quantities of product can be synthesized. However, when a large flexibility is necessary, e.g. when designing a new protein the best approach is probably a combination of the two techniques. Chemical synthesis is first employed to establish a rough version of the desired molecule. The information gained at that stage is used to prepare a genetic design of the protein. The finishing touches might then be put to the protein using site-directed or cassette mutagenesis.

7.1. BIOLOGICAL PRODUCTION OF PROTEINS

Initially, interest in genetic engineering was focused on production of pharmacologically highly active polypeptides, where the demands for the amount of proteins were low, but where the purity was essential. To a great extent, interest has already turned over to bulk scale production of proteins, e.g. production of enzymes for biotechnological processes. This in term often requires among other things:

- (a) Use of genetically stable bacteria
- (b) Novel protein design

7.1.1. Use of genetically stable bacteria

The demand of genetically stable production organisms is mainly caused by the requirement of continuous fermentation. For instance, if a mutant arises which has lost the recombinant plasmid, the mutant will frequently grow faster and may quickly become predominant in the culture because it is more fit of chemostat survival. This is due to the fact that an increase in the level of expression often leads to a concomitant reduction in cell growth.

Naturally occurring plasmids are frequently stably maintained because they contain a partitioning function *par* which ensures that they are accurately segregated at each cell division. However, in many high copy number plasmids such as pBR 322, the *par* region is deleted. This implies that under certain growth conditions, such as rapid cell growth, cells without plasmids may arise (Nugent *et al.*, 1983). Usually, this problem is solved by maintaining antibiotic selection but this is not desirable for large scale culture because of its high cost and risk of contamination of the final product. Therefore, an attractive method of stabilization is the introduction of the *par* region on the vectors (Skogman *et al.*, 1983).

An alternative strategy to counter segregative instability is to reduce or even stop cell growth by different methods (see Chapter 3). Besides the advantages in handling and fermenting brought about by immobilization (entrapment) of bacteria, this procedure can also lead to stabilization in the absence of a selection pressure (de Taxis du Poët *et al.*, 1986).

7.1.2. Protein design

There are at least three desirable properties, a trinity, of the final protein product that should be

fulfilled, in order to simplify its production.

- (a) The protein should be **secreted** to the growth medium or at least to the periplasmic space (Chapter 3.3.). This obliterates the need of cell disintegration and simplifies protein purification.
- (b) The protein should be purified through a **one step purification** protocol, where an affinity column might be coupled on-line with the fermentor. Immunosorption appears attractive but is limited to a small scale. Addition of an "affinity tail" to the polypeptide chain appears more attractive.
- (c) The protein should be easily **monitorable**. Many proteins are assayed for by immunological methods which require access of specific antibodies as well as they tend to be time-consuming. The use of an enzyme label created by fusion of an enzyme gene to the structural gene in question appears attractive as it allows for continuous registering of product formation during fermentation.

The properties (b) and (c) involve the addition of a polypeptide fragment to the protein which as such is less desirable since the total product formation then will be diminished. This then requires that the number of additional amino acid residues should be kept to a minimum.

Affinity tails employed so far, such as protein A (Nilsson *et al.*, 1985) and beta-galactosidase (Ullmann, 1984), tend to be too large. Smaller additions appear more appropriate. For instance, tails of five arginine residues (Smith *et al.*, 1984) or four cysteine residues (Bülow and Mosbach, 1985) have proved useful in this respect using ion exchange chromatography and covalent chromatography, respectively. Another recent approach involves the addition of His-Trp to the N-terminal of the protein which thereby can be purified by immobilized metal ion affinity chromatography (Smith and Pigeon, 1986).

Furthermore, these affinity tails should be removable especially in the case of pharmacological compounds. For instance, a polyarginine in the COOH-terminal can be removed by carboxypeptidase A. Otherwise a specific amino acid residue (or residues) in the connecting region between the tail and the protein must be used. For instance, cyanogen bromide can be employed to cleave at methionine residues and trypsin at arginine or lysine residues.

Use of an enzyme label, as suggested in (c), is easy to accomplish, but it normally involves the

addition of a large polypeptide chain. The preparation of artificial small (and stable) enzyme-like catalysts as outlined in Chapter 5 appears highly attractive in this respect. However, preferably claims (b) and (c) should be fulfilled employing the same peptide chain.

7.2. BIOLOGICAL SYNTHESIS OF POLYPEPTIDES

Biological synthesis of polypeptides can be used to create modified or novel preparations with new features. In this study, emphasis has been devoted to prepare bifunctional proteins employing gene fusion. The physical properties of these artificial bifunctional enzymes have only been studied *in vitro*. However, interesting effects can also be found *in vivo*, for instance, a more efficient utilization of lactose might be found in those *E. coli* having a beta-galactosidase/galactokinase hybrid enzyme (cf. Srere and Mosbach, 1974).

In principle, using this approach any genes can be ligated, but the critical point is whether the resulting hybrid protein will fold sufficiently well to yield autonomous domains. For instance, the carboxyl- or amino-terminal ends might be buried in the interior of the protein. However, in most cases the ends are located on the protein surface due to the charge carried by the amino and carboxyl groups, respectively (Wodak, personal commun.). More importantly and also more probable is the involvement of these ends in subunit interactions or even in the active center which thereby obliterate many potential fusions. Another important assumption is that proteins in general will be forgiving of attempts of modification. This view is supported by the apparent plasticity of proteins. For instance, from mutational studies it is known that many amino acid changes are silent and have little or no effect on the functionality of the protein.

Bifunctional enzymes prepared by gene fusion or protein fusion will not be applicable only to enzyme sequences as discussed in Chapter 4. A wide variety of other systems can easily be envisaged. Four systems will be discussed:

- (a) cyclic enzymic reactions
- (b) enzyme regulation
- (c) coenzyme dependent reactions
- (d) design of artificial catalysts

7.2.1. Cyclic enzymic reactions

A special case of sequentially acting enzymes is those involved in cyclic reactions. Frequently,

when these enzymes are in close proximity to each other a higher steady-state rate can be detected. This can be ascribed to a higher local concentration of the intermediates in the vicinity of the enzymes which thereby renders the cycle more efficient. A potential application can be found in the area of biochemical analysis. Substrate recycling can increase the sensitivity dramatically. For instance, in L-lactate determination using the enzyme thermistor, the combination of lactate oxidase with lactate dehydrogenase and catalase resulted in an enhancement of sensitivity up to 1000-fold compared to lactate oxidase and catalase (Scheller *et al.*, 1985).

7.2.2. Enzyme regulation

When enzymes are arranged in proximity to each other, a product from one enzyme, but not necessarily a substrate for a neighbouring enzyme, may influence the activity of the latter. Such systems might be common *in vitro* to regulate metabolic events. For instance, pH changes may control enzymic activities in the living cell. A model system comprising co-immobilized glucose oxidase, hexokinase and trypsin has thus been employed to study the effect of protons generated by trypsin in its hydrolysis of an ester. The ratio of glucose converted by hexokinase and glucose oxidase, respectively, could be controlled by the esterolytic activity of trypsin (Gestrelus *et al.*, 1973). Similar systems can be prepared by gene fusion.

7.2.3. Coenzyme dependent enzymes

Many reactions of potential practical interest catalyzed by enzymes require the participation of expensive coenzymes such as NAD(H). Methods for their reuse, based on retention and regeneration, have been studied extensively up to date. The actual regeneration can be accomplished by different approaches; chemical, electrochemical, photochemical and enzymic. Of these, use of a recycling enzyme is at present the preferred route.

Different techniques have been employed to solve the retention problem. For instance, Wichmann *et al.* (1981) have covalently coupled NAD(H) to poly(ethylene glycol). The biocatalysts, enzyme and polymer-NAD, are due to their size, confined within a membrane reactor with the aid of an ultrafiltration membrane, while substrates and products can freely diffuse in and out. For instance, the technique has been used to produce amino acids from keto acids with leucine dehydrogenase and alanine dehydrogenase. Formate dehydrogenase is then employed to regenerate the coenzyme.

Another approach is to fix the coenzyme directly on the enzyme. Thus, Månsson *et al.* (1978) prepared a soluble complex with a NAD analogue covalently coupled to horse liver alcohol

dehydrogenase. Each subunit carried approximately two NAD molecules per subunit. One of these had such a position that it could interact with the active site. Using this method the coenzyme will be randomly coupled to the enzyme. It would be highly desirable if the coenzyme analogues could be coupled at a specific site on the enzyme, which also should allow for a correct orientation in relation to the active site. On the protein surface there are several different "reactive" amino acid residues available for coupling such an analogue. COOH- and NH₂-groups (lysine, aspartic acid or glutamic acid residues) are convenient for coupling but proteins usually exhibit a preference for those charged groups on the surface and addition of more groups or removal of undesirable by site-directed mutagenesis do not appear to be an advisable method as a **site-specific** coupling of the coenzyme to the protein would be less probable.

An attractive alternative candidate is cysteine which proteins normally have few of. Moreover, two main alternative coupling procedures exist, reversible (S-S bridge) and irreversible. Extra cysteines can be added at the appropriate sites by site-directed mutagenesis, especially if the three-dimensional structure is known. An alternative approach is to add a flexible tail carrying a few cysteines, which is able to swing in and out of the active site. Thus, the galactokinase gene was modified by introducing a short DNA fragment coding for four additional cysteine residues. The gene product, cys-galactokinase, purified from *E.coli* was allowed to react with an ATP analogue, having an aminohexyl spacer with a maleimide group at the end. The resulting enzyme complex, ATP-cys-galactokinase was able to carry out the normal phosphorylation of galactose without the addition of soluble ATP. Moreover, the ATP moiety could be regenerated by pyruvate kinase, thus giving an enzyme system without the requirements of additional cofactors (Gemeiner *et al.*, to be published).

7.2.4. Artificial enzyme-like catalysts

Advances in our ability to manipulate genes have provided the tools necessary for preparing artificial enzyme-like catalysts, but the theoretical knowledge in protein biochemistry still lags behind our technical capabilities. Nevertheless, an enzyme can be viewed upon as composed of two distinct moieties, a substrate binding domain and a catalytic domain. The design of a region with high affinity for the desired substrate will probably be the most difficult part.

A simple rescue, which easily can be envisaged, is the fusion between an antibody with the appropriate substrate specificity and a "swinging arm" containing the catalytic groups. Another

feasible approach would be to construct a flexible polypeptide chain, which can be chemically cross-linked in the presence of an appropriate substrate or preferably a transition state analogue to stabilize an efficient structure.

Primarily, interest will focus on hydrolytic enzymes, since the rules governing catalysis are known for many of these enzymes. As the mechanism underlying catalysis of other enzyme classes will become revealed, synthetic catalysts mimicing these can be prepared. No doubt, gene fusions will then be an important tool to identify and purify the artificial enzyme-like preparation as outlined in paper VI.

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"Man lär aldrig förgäves inte ens strunt".

Hjalmar Bergman

LIGATION OF RESTRICTION ENDONUCLEASE-GENERATED DNA
FRAGMENTS USING IMMOBILIZED T4 DNA LIGASE

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T4 DNA ligase was covalently coupled to Sepharose 4B using 2,2,2-trifluoroethanesulfonyl chloride activation. The immobilized DNA ligase could catalyze the joining of restriction endonuclease-generated DNA fragments with sticky ends as well as blunted ended DNAs. Immobilization provided an increased stability. At 40°C, the immobilized ligase remained active for at least three months. Nucleic acid synthesis and *in vitro* DNA recombination should be the main fields of application for such immobilized DNA ligase.

INTRODUCTION

DNA ligases have become indispensable reagents for *in vitro* DNA recombination (1). The unique ability of T4 DNA ligase to catalyze the joining of DNA fragments with "sticky ends" as well as blunted ended DNAs has made this enzyme the most extensively used ligase in nucleic acid research (2,3). The enzyme acts by transmuting the high-energy pyrophosphate linkage of ATP into a phosphodiester bond between the 5'-phosphoryl and 3'-hydroxyl termini of DNA.

Immobilization of enzymes and other biomolecules is a widespread and well-known technique in biochemistry (for review see (4)). Also in the "cloning technology", the use of immobilized enzymes should be advantageous, as it allows easy removal of the enzymes from the reaction mixtures as well as their subsequent reuse. In this report we wish to describe the immobilization of such an enzyme, T4 DNA ligase, to Sepharose using the CNBr method as well as the recently described procedure involving 2,2,2-trifluoroethanesulphonyl chloride (tresyl chloride) (5). The latter method was chosen since stable bonds are formed between the matrix and the protein and also because coupling can proceed under mild conditions. Apart from the practical advantages offered by immobilization, such preparations should also provide an insight into questions relating to enzyme mechanism involved in DNA ligation, as discussed later.

MATERIAL AND METHODS

Enzymes: T4 DNA ligase and restriction endonucleases *EcoRI* and *SmaI* were all obtained from Boehringer Mannheim. *TaqI* was purchased from Bethesda Research Laboratories Inc.

DNA's: The plasmid pBR 322 was purified from cleared lysates by centrifugation to equilibrium in CsCl-ethidium bromide gradients (6). Lambda DNA was purchased from P-L Biochemicals Inc.

Tresyl chloride activation of Sepharose: Activation of Sepharose 4B (Pharmacia) was done according to Nilsson and Mosbach (5). The wet Sepharose was transferred to dry acetone by washing with 10 gel volumes of distilled water, 30:70, 60:40 and 80:20 of acetone:water, 2 x 10 volumes of acetone and finally with 3 x 5 volumes of dry acetone (dried over molecular sieve, 100 g/3 l acetone). In a typical experiment 10 g moist Sepharose was then transferred to a beaker containing 1 ml dried acetone and 0.4 ml pyridine. During magnetic stirring 0.2 ml tresyl chloride (Fluka AG, Buchs, Switzerland) was added dropwise for 1 min. After reaction for 10 min. at room temperature the gel was washed with 2 x 100 ml acetone and then gradually transferred back to 1 mM HCl by reversing the washing scheme described above using 1 mM HCl instead of water. The activated gel was stored at 4°C in 1 mM HCl and samples were taken when required during a period of one month.

The degree of activation was determined by elemental analysis of sulphur.

CnBr activation: CnBr activation of Sepharose 4B was done according to March et al. (7) using dimethylformamide instead of acetonitrile. The reactive cyanate groups were determined by the method described by Kohn and Wilchek (8). The activated gel was used immediately.

Enzyme immobilization: 40 U of T4 DNA ligase were dissolved in 0.1 M NaH_2PO_4 , pH 7.5; containing 1 mM EDTA; 50 μM ATP; 10 % glycerol to a final volume of 200 μl and kept at 4°C. A wet tresylated or CnBr activated Sepharose preparation was washed with the above cold phosphate buffer, gently sucked dry, and 100 mg moist gel was thereafter transferred to the enzyme solution. The resulting gel slurry was mixed "end over end" at 4°C for 16 hrs.

Residual reactive tresyl groups on the Sepharose were removed by adding 0.1 M dithiothreitol or in the case of CnBr, by adding 0.1 M 2-mercaptoethylamine (2 hrs, 4°C). After coupling, the gel was washed free of uncoupled enzyme by washing four times in 0.1 M NaH_2PO_4 , pH 7.5; 1 mM EDTA; 10 mM dithiothreitol (DTT); 0.5 M NaCl followed by two washes in 0.1 M NaAc, pH 5.0. Finally the gel was equilibrated in 66 mM Tris-HCl, pH 7.6; 6.6 mM MgCl_2 ; 10 mM DTT; 10 % glycerol and stored at 4°C.

Enzyme activity determinations: The DNA ligase activity was determined by the ^{32}PPi exchange assay devised by Weiss et al. (9). One unit is the enzyme activity which exchanges 1 nmol ^{32}PPi into Norit adsorbable material within 20 min. at 37°C.

DNA joining: The reactions were carried out in volumes ranging from 30 to 300 μl containing 20 $\mu\text{g/ml}$ DNA; 66 mM Tris-HCl, pH 7.6; 6.6 mM MgCl_2 ; 10 mM DTT; 500 μM ATP; 30-50 mg moist DNA ligase gel. The gel slurry was incubated by gentle agitation at 20°C in 1.5 ml plastic tubes. After 16-18 hrs the immobilized ligase was removed by centrifugation.

Agarose gel electrophoresis: Performed on a horizontal slab gel of 1 % agarose in 0.05 M Tris-acetate-EDTA buffer (pH 7.8).

RESULTS

Catalytic properties: As substrates for our study two standard DNAs were used; plasmid pBR 322 and lambda DNA which had been cleaved with the re-

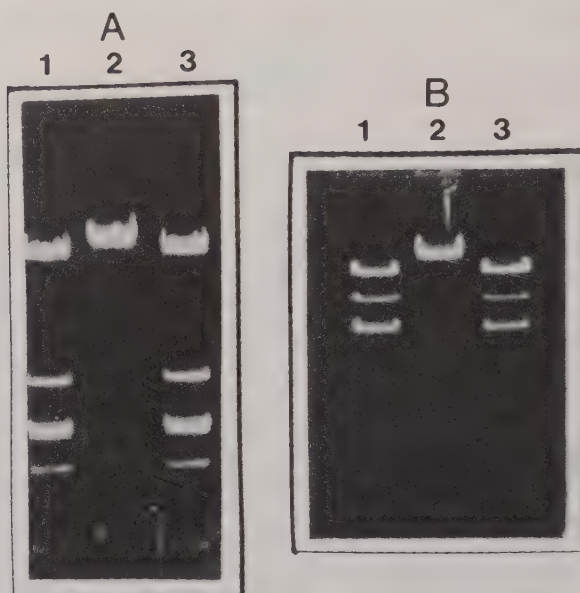


Fig. 1. Ligation/recut assay of *EcoRI* (panel A) and *SmaI* (panel B). Lambda DNA was used as substrate. Lane 1, 1.0 μ g lambda DNA cleaved with respective restriction enzyme; lane 2, ligated products (1.0 μ g) of immobilized ligase; lane 3, 1.0 μ g ligated lambda DNA recut with the same restriction enzyme.

striction enzymes *EcoRI* and *TaqI* to generate fragments with "sticky ends". Blunted ends, i.e. termini without any single stranded overlaps, were obtained by cleaving lambda DNA with *SmaI*. The ligated products obtained with the immobilized ligase after incubation over night at 20⁰ C were subjected to electrophoretic separation on agarose gels. As shown in figure 1, the immobilized ligase could effectively ligate both blunted ends as well as DNAs with sticky ends. In order to check the nucleotide integrity after ligation an aliquot was recut with the same nuclease as previously used (figure 1). No change of the typical cleavage patterns were obtained, indicating that immobilization of T4 DNA ligase does not interfere with the normal catalytic properties of the soluble enzyme.

Coupling efficiency: Immobilization of DNA ligase using tresyl chloride was studied under various conditions. As seen from Table 1, the coupling yield was dependent upon the degree of activation. Coupling efficiency for the alternative CNBr gels with the same degree of activation (i.e. tresyl and cyanate groups, respectively) appeared to be almost identical, i.e. 14-16 %. Linkage between the protein and the matrix is believed to be through amino

Table 1. Coupling efficiency for T4 DNA ligase at different degrees of tresyl chloride activation

Amount of tresyl chloride added in the activation step ($\mu\text{mol/g}$ moist gel)	Amount of tresyl groups formed on the gel after activation ($\mu\text{mol/g}$ moist gel)*	Amount of T4 DNA ligase added (U/g moist gel)	Enzyme activity yield (%)
23	3.3	100	6.3
90	15	100	14.7
180	25	100	9.4
23	3.3	200	7.7
90	15	200	8.7
180	25	200	17.1
90	15	400	13.1
180	25	400	16.5**
230	35	400	11.0
180	25	400 (no ATP added)	5.5

* determined by elemental analysis of sulphur

** as described in Material and Methods

or mercapto groups on the protein. The reactive lysine residue responsible for the ATP binding of the ligase was found to be critical in the immobilization step. If this amino acid residue was not protected through adenylation, the coupling yield was decreased substantially (Table 1).

Stability: T4 DNA ligase in its soluble form is a labile enzyme. At 20° C, i.e. a normal temperature used for DNA ligations, the enzyme loses most of its activity within 24 hrs. As shown in figure 2, stability was greatly improved upon immobilization (tested by the pyrophosphate exchange assay). When stored at 4° C, the immobilized enzyme remained active for at least three months, during which time it was assayed repeatedly for DNA ligation. Immobilization by CNBr was found to give a less stable product having a long-term stability of about one month when stored at 4° C.

Diffusional limitations: Compared with the soluble DNA ligase longer incubation times were necessary for the immobilized enzyme. Dealing with macromolecular nucleic acids, a complete ligation required incubation for 16-18 hrs for the immobilized system while the soluble only needed 1-2 hrs (figure 3). Most likely diffusional hindrance is responsible for the observed differences. However, the reaction rate should not be a critical step in recombinant DNA work as long as the ligation time does not exceed the normally used overnight period.

DISCUSSION

The most commonly used procedure for coupling enzymes to agarose gels involves activation by CNBr. However, the linkages formed are known to be labile and

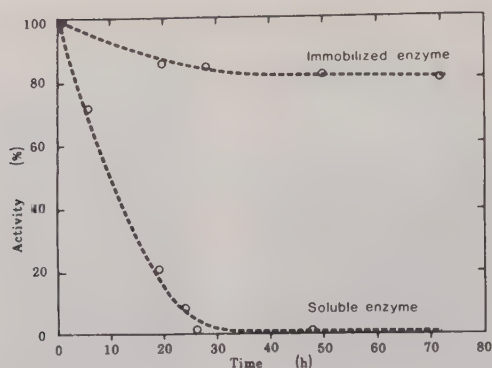


Fig. 2. Stability of soluble and immobilized T4 DNA ligase. 0.5 U of the enzyme was incubated at 20° C in 66 mM Tris-HCl, pH 7.6; 6.6 mM MgCl₂; 10 mM DTT. Activities were determined by the pyrophosphate exchange assay.

additional charged groups might be introduced on blocking the activated matrix causing non-specific adsorption (10). As shown for the immobilized T4 DNA ligase, the tresyl chloride activated gels give rise to preparations of increased stability over those of CNBr treated gels.

We have also tested other enzymes that are used in "cloning research", such as alkaline phosphatase for dephosphorylation of DNA and the restriction endo-

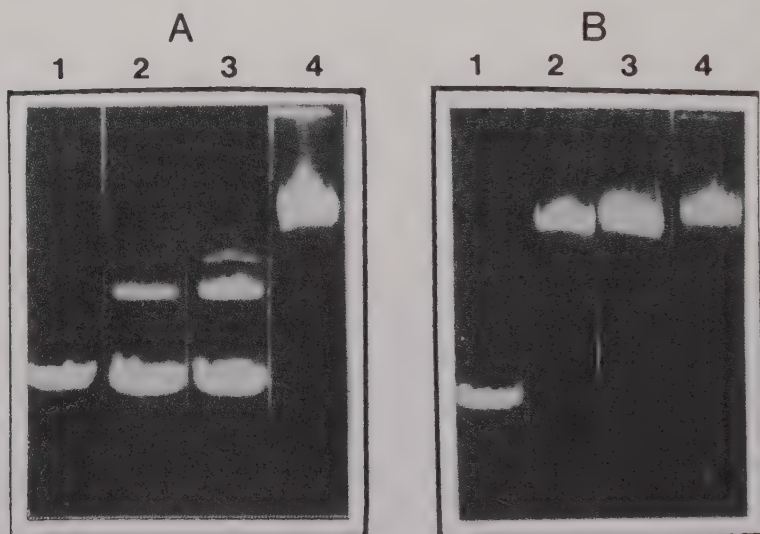


Fig. 3. Comparison of the kinetic properties of immobilized (panel A) and soluble (panel B) DNA ligase. Each reaction mixture contained 5 µg pBR 322 cleaved with *Eco*RI. Aliquots were removed at the following times: lane 1, 0h; lane 2, 1h; lane 3, 2h; lane 4, 16h.

nucleases *EcoRI* and *TaqI*. They all yielded active preparations, however the recovered activity of the restriction enzymes was comparatively low. This was the case both for tresyl chloride as well as for CNBr activated Sepharose (the latter procedure had previously also been applied to the restriction enzymes (11)). This could be explained by the fact that essential lysines are involved in general electrostatic interactions with phosphates in the DNA backbone (12, 13). Apparently, coupling with CNBr or tresyl chloride interferes with critical elements of the active sites of these enzymes. This problem might, however, partially be circumvented by co-immobilizing the enzyme in the presence of a suitable DNA molecule or by immobilizing the enzyme on a matrix with a very low degree of activation.

Immobilized enzymes should greatly simplify work with nucleic acids as the coupled enzymes can easily be removed from the reaction mixtures (no phenol extraction is needed) and thus be reused. Furthermore, when used in enzyme columns in large scale DNA work these reagents have great potential. For instance, oligonucleotides prepared by chemical means could subsequently be ligated on preparative scale using an immobilized DNA ligase column.

Besides the practical advantages offered by immobilizing T4 DNA ligase, we anticipate that this technique will also find application in mechanistic studies of this enzyme. For instance, the actual mechanism of blunt end ligation is still unknown. What we do know is that higher enzyme loading is required to carry out the reaction to completion. It is likely that oligomeric forms of the ligase are involved in the joining of blunted ends as opposed to the involvement of the monomeric form of the enzyme in joining cohesive ends or sealing a nick. Recently, it was found that adenylation of the DNA ligase could affect its oligomerization (14). By immobilizing the ligase at different ATP concentrations affecting the monomer-oligomer ratio, valuable conclusions from the kind of observed activity may be drawn.

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Formation of proinsulin by immobilized *Bacillus subtilis*

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There has been an increasing interest in the use of immobilized cells for the production of pharmaceuticals as well as for products such as high fructose syrup or ethanol¹. Some of these compounds are now produced on an industrial scale² whereby the cells are used in a resting or growing state or in a nonviable form as natural carriers of the enzyme(s) involved in the synthesis. The advantages of immobilized cell technology should also apply to microorganisms modified by recombinant DNA techniques to produce a variety of eukaryotic proteins such as hormones. We describe here the properties of immobilized *Bacillus subtilis* cells carrying plasmids encoding rat proinsulin. Cell proliferation normally coupled to DNA replication is undesirable in immobilized cell systems as 'clogging' of the system occurs due to cells growing outside the beads. Therefore, different ways were investigated to inhibit cell division while allowing continued protein synthesis. We found that the addition of certain antibiotics in the growth medium, such as novobiocin which inhibits DNA replication³, fulfills these requirements, allowing proinsulin synthesis and excretion to take place over a period of several days.

The strain used for these experiments was *B. subtilis* BS273 which comprises the host strain SL438 (provided by P. Piggot) carrying plasmid pPCB6. Plasmid pPCB6 was isolated by inserting a fragment specifying rat preproinsulin⁴ into a 4.3-kilobase sequence encoding the penicillinase of *B. licheniformis* which was cloned on plasmid ppen4300⁵ (provided by H. Schaller). A 350-base pair DNA fragment encoding rat preproinsulin was obtained by digesting plasmid p218.CB6 with the restriction

endonuclease *Pst*I. This fragment was inserted at the *Pst*I site which occurs within the DNA sequence encoding the NH₂-terminal signal peptide of *B. licheniformis* penicillinase⁵. Ligation of the DNA fragments and selection of the recombinant plasmids by transformation were done using methods previously described⁶. Plasmid pPCB6 thus encodes the first 15 amino acids of the penicillinase signal followed by the signal sequence of rat preproinsulin.

A number of support materials including alginate, polyacrylamide and agarose were tested for their ability to entrap the cells as well as to release proinsulin. Agarose 2% (w/v) was

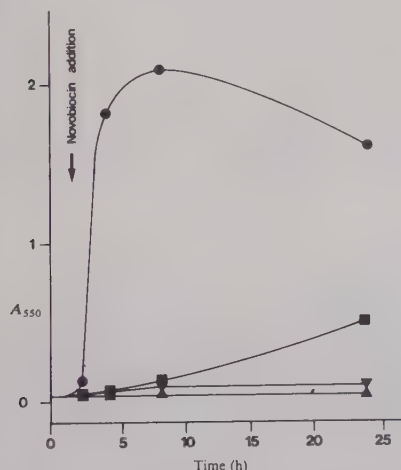


Fig. 1 The effect of novobiocin on growth of free *B. subtilis* BS273 cells. The cells were grown at 37 °C with vigorous shaking (150 r.p.m.) in L-broth supplemented with 12.5 µg ml⁻¹ tetracycline. Novobiocin concentration used were 0 µg ml⁻¹ (○), 2 µg ml⁻¹ (□), 5 µg ml⁻¹ (▼), and 8 µg ml⁻¹ (▲).

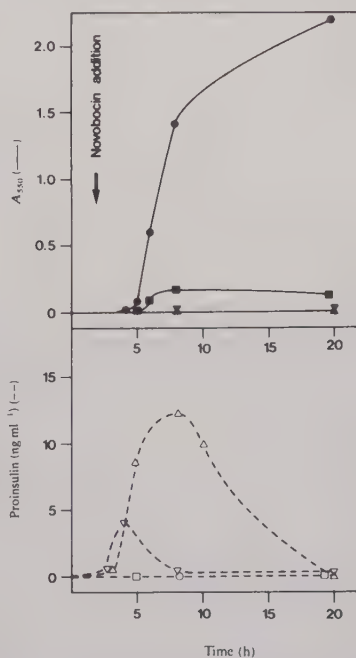


Fig. 2 *B. subtilis* cells were grown in 25 ml medium as described in Fig. 1 and collected during exponential growth at A_{550} 1.0. The cells were centrifuged (5,000g) and washed twice with phosphate-buffered saline (PBS). Twenty-five ml of the cell suspension was mixed with an equal volume of melted 4% (w/v) agarose (type VII, Sigma), kept at 42 °C, and poured into a 1-litre beaker containing 500 ml soya oil (prewarmed to 37 °C) under vigorous stirring. When the formed beads were 100–300 µm in diameter the suspension was cooled to below the setting temperature of agarose. After the beads had solidified, 300 ml PBS was added, stirring ceased, and the immobilized preparation was allowed to settle overnight at room temperature. Subsequently, the oil layer was decanted and discarded (the final droplets were removed with a pipette). Finally, the beads were filtered on a nylon net (250 mesh) and washed with PBS. The immobilized *B. subtilis* preparation (5 g, wet weight) was added to 20 ml of the above medium in 100 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker (150 r.p.m.) at 37 °C. The A_{550} and the proinsulin content of the medium were determined by removing 1 ml samples. After incubation for 2 h novobiocin was added to the medium. The rat proinsulin produced was quantified by liquid radioimmunoassay⁷. Proinsulin formation (----) was determined at different novobiocin concentrations: 0 µg ml⁻¹ (○), 2 µg ml⁻¹ (□), 5 µg ml⁻¹ (▼), and 8 µg ml⁻¹ (▲). The corresponding absorbances (—) were determined at 550 nm.

Table 1 Cell growth inside agarose beads with and without addition of novobiocin

Hours	Cell number per g immobilized beads (wet weight) Without novobiocin	With novobiocin
0	2×10^8	—
2	2.2×10^8	
5	4.2×10^8	4.3×10^8
20	8.9×10^8	4.2×10^8

most effective as it allowed rapid release of entrapped ^{125}I -labelled insulin; in addition it is non-toxic. Agarose beads (2 mm diameter) released 80% of the entrapped ^{125}I -insulin after 2 hours' incubation in 0.1 M Tris-HCl (pH 7.5) whereas polyacrylamide (10% w/v) or calcium alginate (2% w/v) beads released only 15% and 20% of the entrapped insulin. The concentration of novobiocin required to arrest growth of *Bacillus subtilis* in suspension cultures was tested and it was found (Fig. 1) that at a concentration of $5 \mu\text{g ml}^{-1}$, growth was inhibited but a similar amount of proinsulin ($7\text{--}10 \text{ ng ml}^{-1}$) was found in the medium as in the untreated control. The *B. subtilis* cells were then included in agarose and formed into minute beads (100–300 μm diameter) using a method in which inert oil acts as suspension medium^{7,8}. The viability of the entrapped cells based on cell growth and oxygen consumption was tested. As seen from Table 1 the number of cells inside the agarose beads increased over a period of 20 h. On addition of $5 \mu\text{g ml}^{-1}$ novobiocin after 2 h of growth, cell division was completely arrested. These figures were obtained by correlating the total protein content found in the immobilized preparations to that found with free cells⁹.

Oxygen consumption of the immobilized preparations was also determined. On average about $0.4\text{--}0.8 \mu\text{g}$ per min per g bead preparation (wet weight) was consumed as determined with an oxygen electrode¹⁰. These measurements were carried out by washing the immobilized preparation on a glass filter and adding 0.1 g of the preparation to 2.9 ml air-saturated medium and oxygen consumption measured. No significant difference was found for the different preparations after growth for 5 and 20 h respectively.

Immobilized *B. subtilis* cells were incubated in L-broth medium supplemented after 2 h of incubation with various amounts of novobiocin. As seen from Fig. 2, at concentrations of 5 or $8 \mu\text{g ml}^{-1}$ no cell growth was found but proinsulin production continued. The fact that the proinsulin levels decreased is probably due to protease action. In the control (no novobiocin added) considerable cell growth was found outside the beads. (Again proteases presumably account for the fact that no proinsulin was detected.)

We then carried out a study on the continuous production of proinsulin using immobilized *B. subtilis* cells in a small model for a continuous stirred tank reactor. Although novobiocin prevented cell growth outside the beads, continuous formation of proinsulin over a period of 80 h took place (Fig. 3). The amount of proinsulin formed was constant in the range $7\text{--}10 \text{ ng}$ per ml of medium ($9,000\text{--}13,000$ molecules per cell) as determined by radioimmunoassay⁴.

This effect of novobiocin, acts by inhibiting supercoiling of DNA catalysed by DNA gyrase and thus DNA replication³ whilst not affecting protein synthesis, indicates the potential usefulness of such an approach to the production of excreted protein by immobilized cells obtained by recombinant DNA techniques. In a preliminary study using the same approach with *B. subtilis* producing α_2 -interferon¹¹ it could be shown that in the presence of novobiocin, cell growth outside the beads was prevented while continuous interferon excretion into the medium took place.

Other antibiotics tested, including nalidixic acid and 6-(p-hydroxyphenylazo)-uracil had similar effects. Thus, as tested with a genetically engineered strain of a periplasmic leaky

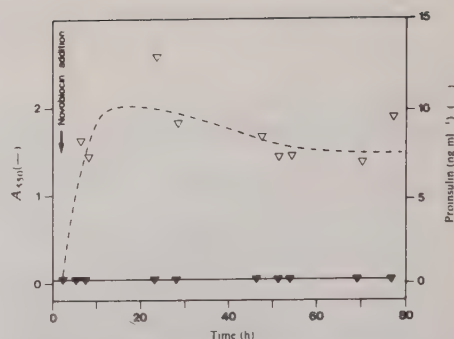


Fig. 3 Continuous formation of proinsulin by immobilized *B. subtilis*. Five grams of the immobilized preparation were added to 20 ml of the medium in 100 ml Erlenmeyer flasks and shaken (150 r.p.m.) at 37°C . Novobiocin ($5 \mu\text{g ml}^{-1}$) was added to the medium after incubation for 2 h. Fresh medium supplemented with novobiocin was continuously added to the small continuous stirred tank reactor at a flow rate of 4 ml h^{-1} . The A_{550} (▽) and the amount of proinsulin (Δ) in the effluent were determined by taking aliquots of 1 ml.

mutant of *E. coli* (EC623; S. Stahl and K.H., unpublished results), addition of 0.1 mM nalidixic acid leading to the inhibition of cell growth, still maintained a production level of proinsulin of 100 ng ml^{-1} found in the medium. Similar results were obtained with free cells. Thus the technique of arresting cell growth by inhibition of cell division with antibiotics while protein biosynthesis/excretion continues should also be useful for conventional fermentation processes using free cells as lesser amounts of cells are required for the same amount of hormones formed.

In the case of immobilized systems using genetically engineered organisms, arresting of cell growth, however, appears to be a prerequisite for hormone production. Provided that protein synthesis can be maintained over long periods of time while controlling cell growth (with novobiocin or nalidixic acid, for example), immobilized systems offer a valuable alternative to conventional fermentation techniques using cells in suspension. Advantages of this technique include: high cell densities in a dilute system; the cells can be protected from damage (possibly also leading to delayed lysis); and no removal of the product from the cells is required. As a further step the on-line coupling of specific affinity columns to an immobilized system can be envisaged to allow rapid isolation of the product.

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"When the dust passes thou wilt see whether thou
ridest a horse or an ass".

Oriental Proverb

Automated Thermometric Enzyme Immunoassay of Human Proinsulin Produced by Escherichia coli¹

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running title

Automated Enzyme Immunoassay of Human Proinsulin

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automated assays

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proinsulin, enzyme immunoassay, enzyme thermistor

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2. To whom correspondences should be sent.

Abstract

We have determined and monitored the production and release of human proinsulin by genetically engineered Escherichia coli cells. Several M9 media samples were analyzed sequentially after centrifugation with the aid of a rapid automated flow-through thermometric enzyme linked immunosorbent assay (TELISA) system. The response time was 7 minutes after sample injection and a single assay was complete after 13 minutes.

Insulin concentrations in the range of 0.1-50 mg/ml could be determined. The TELISA method correlated well with conventional radioimmunoassay determinations. Standard curves were reproducible over a period of several days even when the immobilized antibody column was stored at 25°C in the enzyme thermistor unit. Thus, immediate assay start up was possible.

INTRODUCTION

Fermentation and cell cultivation are generally characterized, evaluated and controlled by monitoring secondary parameters such as pH, O_2 and CO_2 .

Various metabolites, such as glucose or ethanol, can be analyzed enzymatically and thus allow a more direct interpretation of the process under study, particularly if the metabolite analyzed is the product of interest. Immobilized enzymes, applied in the form of enzyme electrodes or enzyme thermistors can nowadays also be employed even on-line (1,2). Thus, the time of the assay, the amount of enzyme used and the risk of contamination are minimized. Furthermore, the use of immobilized enzymes is well suited for automation due to increased operational stability.

Many of the current products of interest are proteins produced by genetically engineered or hybridoma cells. We have investigated the production of such proteins by immobilized cells (3,4). Specific and sensitive quantitation of such proteins is generally achieved immunologically by such methods as radioimmunoassay (RIA) or enzyme linked immunosorbent assay (ELISA) (5,6). The latter incorporates antibodies immobilized through adsorption to a solid phase which in practice has increasingly meant the inner surface of sample wells of specifically manufactured 96-well microtitre plates.

If the antibodies are covalently bound to a solid phase, the immunosorbent can be used repeatedly. If the solid phase is beaded agarose the immunosorbent can be packed into a column and employed under non-equilibrium, flow-through conditions which, in turn, would result in rapid response times. To ensure accuracy and consistency, strict isokinetic conditions would be required. This is best achieved in an automated thermostatted system. The enzyme activity of the enzyme labelled antigen (or antibody) used in the ELISA method is generally measured spectrophotometrically. However, the enzyme activity can also be measured thermometrically. The latter technique has a potential advantage especially when dealing with fermentation media which may, by their very nature, contain several constituents which might interfere with spectrophotometrical measurements. Thus, an enzyme thermistor together with the system described above (not automated) for the determination of human serum albumin was employed and coined TELISA (thermometric enzyme linked immunosorbent assay) (7). Later, the TELISA method was used for the determination of insulin in phosphate buffered saline (PBS) (8). In this communication we describe an automated TELISA system for analyzing samples of M9 media

containing human proinsulin produced by genetically engineered Escherichia coli cells. The system and results are described and discussed below.

MATERIALS AND METHODS

Chemicals

Anti-porcine insulin serum was purchased from Miles (Rehovot, Israel). Porcine proinsulin and ^{125}I -porcine insulin were obtained from Novo (Copenhagen, Denmark). Beef insulin, horse radish peroxidase (type VI) and 4-aminoantipyrine were acquired from Sigma (St. Louis, Mo., U.S.A.). Sephacryl S-200 (SF) and Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). 2,2,2-Trifluoroethanesulfonyl chloride (tresyl chloride) was obtained from Fluka (Switzerland). Tresyl-activated Sepharose 4B is commercially available from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, FRG).

Apparatus

The enzyme thermistor was developed and constructed at this department and has been aptly described (2). The timer and autosampler were also constructed at our department. The timer consists of two clocks, a cycle timer and an injection timer, together with an HPLC valve (Rheodyne, Santa Rosa, CA, U.S.A.). Alternatively, a programable controller (Hizac D28, Hitachi, Japan) was used to control the sample injection and washing cycles indicated in Fig. 2.

Preparations

Insulin antibody-Sepharose 4B: The anti-insulin serum was first affinity purified against beef insulin. Five mg beef insulin per g wet gel was coupled to Sepharose 4B activated with 6 ml tresyl chloride per g wet gel (9). The anti-insulin serum was applied to a column (2.5 g wet gel) containing the insulin bound Sepharose 4B. After washing the column with 50 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) until A_{280} was 0.00, the antibodies were eluted with 0.2 M glycine-HCl, pH 2.2, with reverse flow. The eluted peak was immediately mixed with an equal volume (1.5 ml) 0.5 M Na_2HCO_3 and dialyzed against 0.2 M Na_2HCO_3 overnight at 4°C.

The purified antibodies (0.2 mg/ml) were then coupled to Sepharose 4B (1 ml/g wet gel) activated with tresyl chloride (6 ml/g wet gel).

Insulin-peroxidase conjugate: The beef insulin-peroxidase conjugate was prepared according to Wilson and Nakane except that after the coupling reaction (see step 5 in reference 10) the conjugate was dialyzed against 0.01 M Na_2HCO_3 (pH 9.5) containing 0.5 g/l NaBH_4 for 2 hours at 4°C (10). Subsequently, the reaction mixture was dialyzed against PBS at 4°C overnight before being chromatographed on Sephacryl S-200. The initial weight ratio of insulin to peroxidase was 1:2.

Fermentation

Escherichia coli EC 703 was obtained from Biogen S.A. (Geneva, Switzerland). The bacterium, W3110i^q, carries a plasmid, ptc 90K8, encoding for human proinsulin. The plasmid was constructed by S. Stahl at Biogen. The prokaryote releases the proinsulin molecule into the media primarily through cell lysis. The cells were cultured at 37°C in 100 ml Erlenmeyer flasks at 200 rpm in 10 ml M9 media containing (g/l) casamino acids (20.0), glucose (2.0) and ampicillin (0.04). The cells were induced with 0.5 mM isopropyl-b-D-thiogalactoside (IPTG) at an $\text{OD}_{550}=1.0$. One ml samples were antiseptically withdrawn at various times during the fermentation. The samples were centrifuged (10,000 g x 10 min) to remove cells before analysis.

Assay procedure

The experimental set-up is shown in Fig. 1. In the experiments described below, both the injection and cycle timers were set at two minutes. First, the autosampler is activated so that the sample syringe advances to the first position on the sampler rack. The injection loop (0.5 ml) is filled with the sample with the help of a peristaltic pump (2.4 ml/min). After approximately 20 seconds the injection timer is activated and the valve is rotated to the inject position so that the contents of the loop is injected into the enzyme thermistor (0.6 ml/min), again with the help of a peristaltic pump. Thus, after one minute the loop is washed with PBS. The enzyme thermistor is constantly set at 25°C. After a total of two minutes the valve returns to the fill position and the cycle timer is activated. During the next two minutes, PBS bypasses the injection loop and flows directly into the enzyme thermistor and washes the immobilized

antibody column. After two minutes the autosampler is again activated and the whole procedure is repeated.

For each fermentation sample determination three test tubes are required on the sampler rack. The first testtube contains 0.5 ml cell-free fermentation sample (or standard in M9 media) plus 0.5 ml insulin- peroxidase conjugate (the stock solution is typically diluted 1:20 with PBS). The second testtube is filled with 1.0 ml of the substrate for peroxidase (2 mM H_2O_2 , 14 mM phenol and 0.8 mM 4-aminoantipyrine in PBS). The heat of analysis upon substrate injection from the antibody bound enzyme labelled antigen is detected by the enzyme thermistor and subsequently observed and measured on the recorder. The third testtube contains 1.0 ml glycine-HCl, pH 2.2 which elutes the insulin-peroxidase conjugate and proinsulin from the immunosorbent, thereby allowing subsequent fermentation sample determinations to be made. Figure 2 shows schematically the principle of the assay procedure.

If a programmable controller or microprocessor is used to control the assay cycle a much simpler procedure can be devised. In that case the sample valve can be used both for sample and substrate introduction as selected by a solenoid valve regulated by the programmable controller which also automatically selects washing (PBS) or eluting (glycine) agents through solenoid valves. The duration of the different events are easily programmed according to the scheme in Fig. 2.

Radioimmunoassay was performed according to L. Heding (11).

RESULTS AND DISCUSSION

The antibodies that were to be immobilized were first purified by affinity chromatography. Elution of the antibody from the immobilized antigen, beef insulin, was accomplished with the same elution buffer used in the assay, 0.2 M glycine-HCl, pH 2.2. From 1 ml anti-porcine insulin guinea pig serum, 0.6 mg of antibodies were obtained.

The antibodies were subsequently immobilized to Sepharose 4B at a concentration of 0.2 mg/g wet gel by the tresyl chloride method, a coupling procedure which we use routinely at our laboratory for immobilizing ligands and enzymes with high yield (12,13). From conjugate binding studies, 51 % of the immobilized antibodies sites were available for enzyme labelled

antigen binding. However, this figure may be higher since in the calculations we have assumed that both antigen binding sites of the antibody are available and that only one insulin molecule per conjugate binds to the antibody. One could envision the binding of a conjugate molecule to one binding site may sterically obstruct the binding of another conjugate molecule to the other antigen binding site of the antibody. Furthermore, since the conjugate contains, on the average, at least two insulin molecules (see below), one conjugate could, in fact, bind to two antigen binding sites.

Horse radish peroxidase (HRP) was conjugated to beef insulin by a modified method of Nakane and Wilson (10). The enzyme-antigen ratio can generally be calculated by comparing the A_{403} and A_{280} values which are given in literature as 22 and 7, respectively, for a 1% (w/v) solution of HRP (14). However, we occasionally found that these values were significantly altered for HRP after conjugation such that $A_{403}(1\%) = 5$ and $A_{280}(1\%) = 5$. The $A_{280}(1\%)$ for insulin was 10 before as well as after conjugation. Using these absorbance values, the HRP:insulin molar ratio of the stock conjugate solution was calculated to be 1:2.5 and contained 0.48 mg/ml HRP and 0.17 mg/ml insulin.

A schematic description of the assay procedure is shown in Fig. 2. The principle of the assay is, in essence, the same as a direct competitive enzyme linked immunosorbent assay (ELISA) but here the enzyme activity being measured thermometrically with the help of an enzyme thermistor. The unlabelled antigen in the sample (standard) is mixed with a fixed amount of enzyme labelled antigen. The mixture is subsequently exposed to the immunosorbent for which they compete. As the amount of unlabelled antigen in the sample increases, the amount of enzyme labelled antigen which binds to the immunosorbent will decrease. The amount of bound enzyme labelled antigen is then determined by measuring the enzyme activity thermometrically when substrate is added. Finally, the unlabelled and enzyme labelled antigen is removed from the immunosorbent with low pH glycine buffer, thereby regenerating the immunosorbent for subsequent assay. Intermittent to each step the immunosorbent, which is situated within the enzyme thermistor unit, is washed with PBS for roughly three minutes. The antigen concentration of the fermentation sample is extrapolated from standard curves determined similarly with known amounts of antigen.

In preliminary experiments the immobilized antibody column was removed from the enzyme thermistor after the assay run and stored at 4°C in PBS and 0.05 % NaN₃. However, a two hour start-up delay was required the next time the column was to be used in order to obtain a stable base line from the enzyme thermistor. Later, it was found that the immobilized antibody column could be stored at 25°C in the enzyme thermistor for a week (in PBS, 0.05 % NaN₃) without significant loss of binding capacity. Thus, insulin determination could be made immediately the next assay day without delay (after flushing with PBS).

Due to the "flow-through" nature of the assay, the antigen (-enzyme conjugate) is only briefly in contact with the immobilized antibody and thus equilibrium conditions are not achieved. Consequently, it is essential that physiochemical parameters, such as temperature, pH, injection volume, time and flow rate, are kept constant to ensure repeatable and consistent results. This is inherent in an automated, thermostatted system as the one described above. As stated previously, the assay resembles a direct competitive enzyme linked immunosorbent assay and thus constitutes the fastest of the solid phase enzyme immunoassays available. The assay takes roughly 13 minutes to complete with the results actually being obtained 7 minutes after sample injection (the last 6 minutes are required for the elution step and base line stabilization).

The assay time could be decreased if, for example, the sample volumes and the immobilized antibody column volume could be decreased. However, the absolute peak heights of the assay would also decrease which would need to be compensated by an increase conjugate concentration and/or an increased immobilized antibody concentration. Increasing the flow rate also reduces the assay time, but again the absolute heat peak heights are diminished and would also need to be offset. The maximum flow tolerance of the matrix must also be considered. For Sepharose 4B the manufacturer recommends a flow rate of 11.5 ml.cm²h⁻¹ and 26 ml.cm²h⁻¹ for Sepharose 4B and CL-4B respectively (15). Alternatively, supports with higher flow tolerance can be employed (e.g. polyacrylamide, controlled pore glass, etc.).

A number of ways were found to increase the sensitivity of the assay procedure. For example, by decreasing the conjugate concentration (which also decreases the absolute heat peak height) or by increasing the temperature of the enzyme thermistor one could increase the per cent temperature change per microgram insulin. At 37°C we could detect 0.1 mg/ml insulin. In

addition, we found that the absolute heat peak height was exponentially related to the conjugate dilution factor as seen in Fig. 3.

Using known amounts of porcine proinsulin in M9 fermentation media, a standard was made as seen in Fig. 4. The TELISA method was then compared to the conventional radioimmunoassay method for determining actual fermentation samples from an Escherichia coli strain that produced and released human proinsulin, as shown in Table I. Fermentation samples were taken at various time points after induction. The optical density at 550 nm was recorded, the samples were then centrifuged to remove free cells and subsequently assayed by the TELISA and RIA methods. The results from the RIA determination were obtained 24 hours after assay onset whereas the TELISA determinations were obtained seven minutes after sample injection. In addition, the RIA method required more manual handling (eg. pipetting steps) than the ELISA method. All the fermentation samples plus the standard samples were assayed simultaneously in parallel with the RIA method whereas the samples were assayed sequentially for the TELISA method. The standard samples could be quantified with the TELISA method prior to fermentation. Furthermore, the fermentation samples had to be diluted 1:1000 for the radioimmunoassay determinations in order that they would lie within the standard curve concentrations. As seen from Table I, the results correlate quite well with each other.

An actual example of how the TELISA response appears on the recorder is shown in Fig. 5. A standard curve of porcine proinsulin was made followed by a triple determination of a fermentation supernatant from Escherichia coli cells which produce and release human proinsulin. These samples were obtained from Biogen S.A. (Geneva, Switzerland). A value of 19.5 mg/ml proinsulin was obtained by the TELISA method compared to 20.0 mg/ml by the RIA method (performed at Biogen S.A.).

In order to substantiate the reliability and stability of the TELISA system, a series of standard curves were run at one and three day intervals. During this time the immobilized antibody column was stored in PBS containing 0.05 % NaN_3 at 25°C in the enzyme thermistor.

Consequently, assays could be performed immediately the next assay day after flushing the immobilized antibody column with PBS lacking sodium azide. The standard curves are shown in Fig. 6. As seen virtually no decline in binding capacity is observed. During an assay run, we observed no deterioration of the conjugate or the substrate, in terms of their ability to produce

reproducible heat peaks, over a period of six hours storage on the sampler.

After a particular immobilized antibody column had been used for a period of time, a "blank" activity could be observed, i.e. when no conjugate had been injected. A feasible explanation to this would be that some of the enzyme labelled antigen has remained bound to the immunosorbent even after glycine elution. Furthermore, we have observed that a gradual decline in binding capacity does occur after continuous use. These two factors limit the lifetime of the immobilized antibody column. Thus, it is of outmost importance that both the "blank" activity and the immobilized antibody column's binding capacity are regularly checked (eg. by making a standard curve). Once unacceptable deterioration of a solid phase is established, a new column should be installed. In general, we have found that a single column gave satisfactory results for approximately two to three weeks.

Lastly, we should mention that when a new column is used for the first time a decrease in the absolute heat peak height was observed for the first couple of assays, after which the assay determinations were stable. A plausible explanation can be that initially some of the immobilized antibodies are irreversibly denatured by the first couple of glycine elution steps.

In conclusion, we have shown that the TELISA method can be employed in practice for analysis of media samples containing human proinsulin produced and released by Escherichia coli cells. No separation steps other than centrifugation were required. It should also be possible to design the assay method for on-line determination if a simple dialysis device together with a conjugate and a sample mixing chamber or coil is introduced into the system. The system was automated and regulated either by a preset timer or a programable controller. The results obtained from the TELISA method correlated well with those obtained by radioimmunoassay. Though the TELISA method does not exhibit the sensitivity that RIA does, the detection limit 0.1 mg/ml was by far adequate for our purposes. The method is however, exceedingly rapid and simple (particularly when automated) compared to the RIA method. For example, the response time is seven minutes for the TELISA method. Another difference between the two methods is that the samples can be assayed sequentially with the TELISA method whereas all the samples are routinely assayed simultaneously with the RIA method because of the complicated nature of the latter procedure. In addition, with the TELISA method the antibodies can be reused since they are immobilized to a solid phase and therefore represents, in principle, a more economical method than the RIA method. Since the specificity of the assay method depends on the antibodies immobilized, the

TELISA method of analysis described here is widely applicable to many other products of interest as long as antibodies to the product are available, including other proteins produced by genetically engineered cells or monoclonal antibodies produced by hybridoma cells. Investigations of the latter are currently under study (O.W. Merten, personal communication).

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FIGURE LEGENDS

- Figure 1:** Experimental set-up for automated TELISA. 1 = autosampler, 2 = timer with valve (A = injection timer, B = cycle timer), 3 = peristaltic pump, 4 = PBS reservoir, 5 = waste, 6= enzyme thermistor, 7 = wheatstone bridge, 8 = recorder.
- Figure 2:** Schematic representation of TELISA procedural steps. (Y) = antibody, (■) = antigen, (E) =enzyme, (S) = substrate, (P) =product.
- Figure 3:** Relationship of heat generated at various conjugate concentrations. Various dilutions of the conjugate were assayed without antigen as described in th text.
- Figure 4:** Standard curve of porcine proinsulin. The conjugate was diluted 1:10 with PBS. The assay procedure is described in the text.
- Table I:** Comparison of RIA and TELISA determinations of human proinsulin production by genetically engineered *E. coli* cells. The conjugate was diluted 1:50 with PBS. The fermentation conditions and the assay procedure are described in the text.
- Figure 5:** Actual response peaks as seen on the recorder. In ascending order, a standard curve of porcine proinsulin was first run with 0, 5, 10, 20, 30 and 40 mg/ml followed by a triple determination of a fermentation supernatant of *E. coli* cells producing human proinsulin. The assay procedure is described in the text. The disturbance in the baseline seen after the temperature peak is caused by solvation effects from the glycine wash.
- Figure 6:** Standard curve variation with immobilized antibody column stored in the enzyme thermistor. (▲) = day 1, (■) = day 2, (●) = day 5. The conjugate was diluted 1:10 with PBS. The assay procedure is described in the text.

Fig. 1

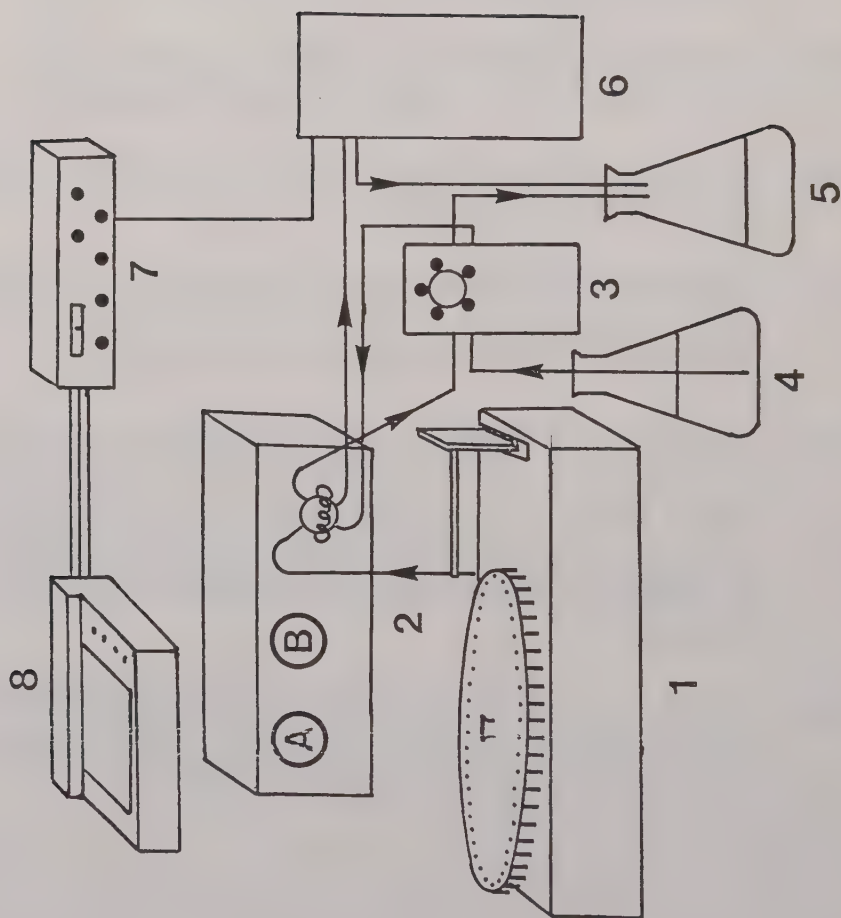


Fig. 2

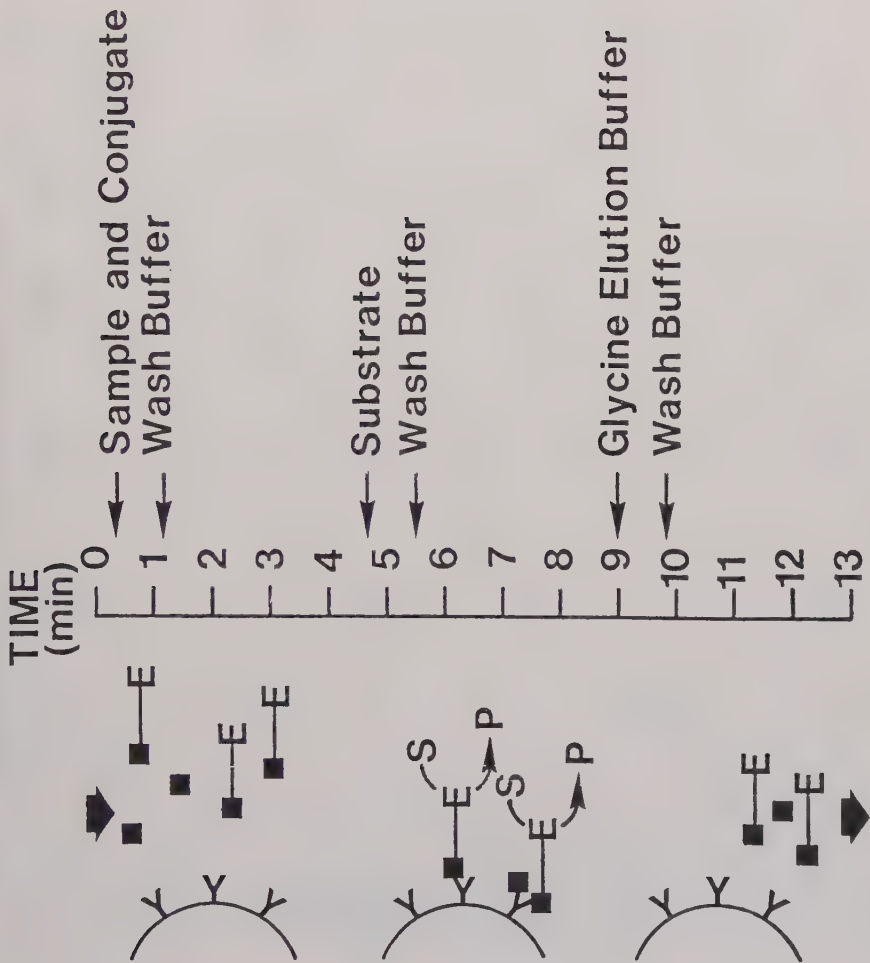


Fig. 3

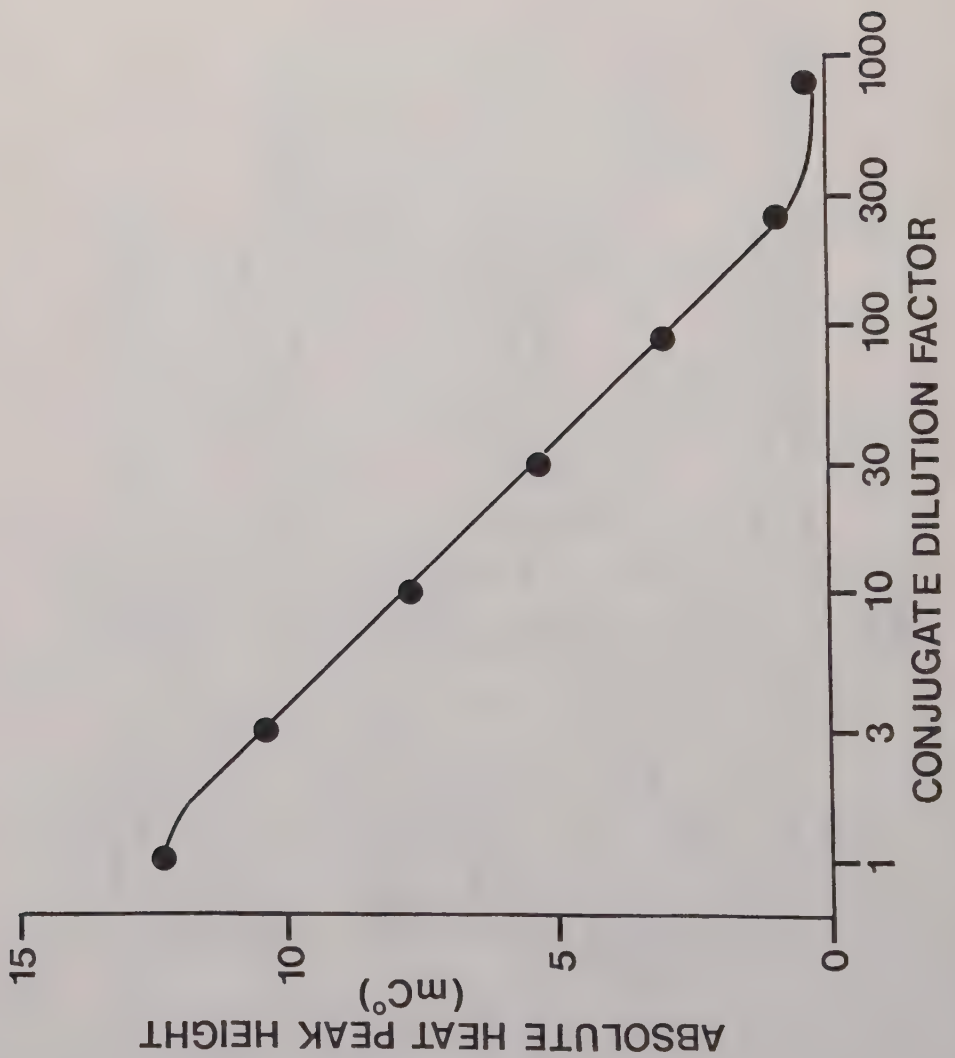


Fig. 4

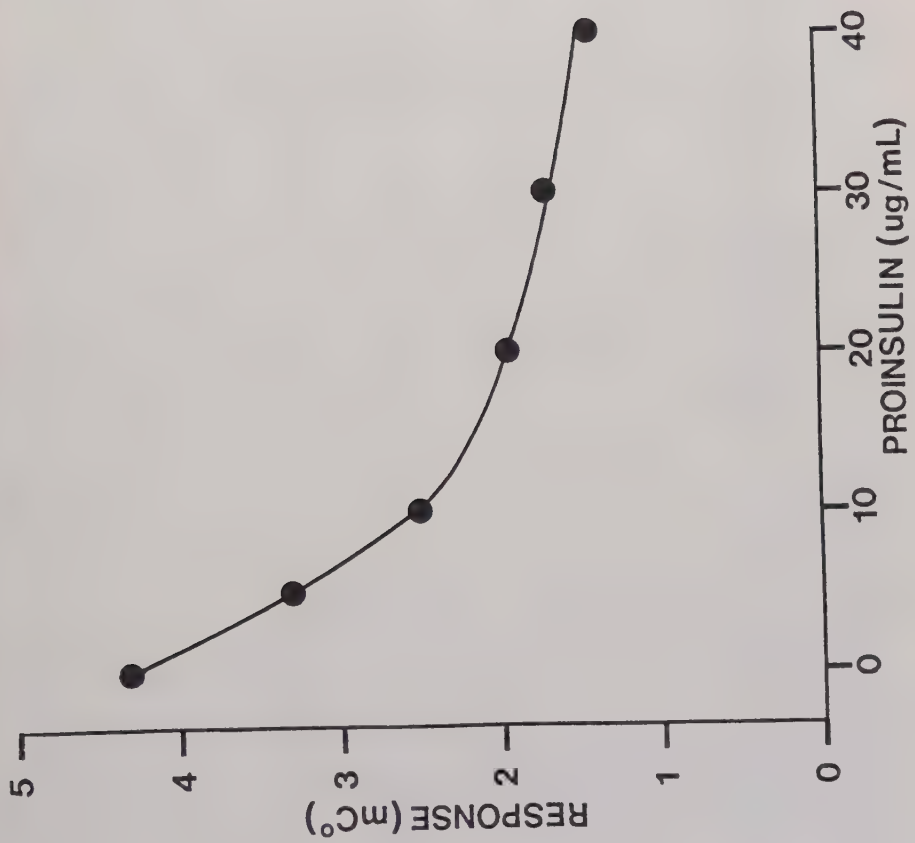


Table I

TIME (hours)	OPTICAL DENSITY (550 nm)	RIA PROINSULIN (ug/mL)	TELISA PROINSULIN (ug/mL)
3	4.0	0.7	0.8
6	4.5	1.7	1.6
11	4.4	2.1	2.4
25	4.4	2.1	2.5

Fig. 5

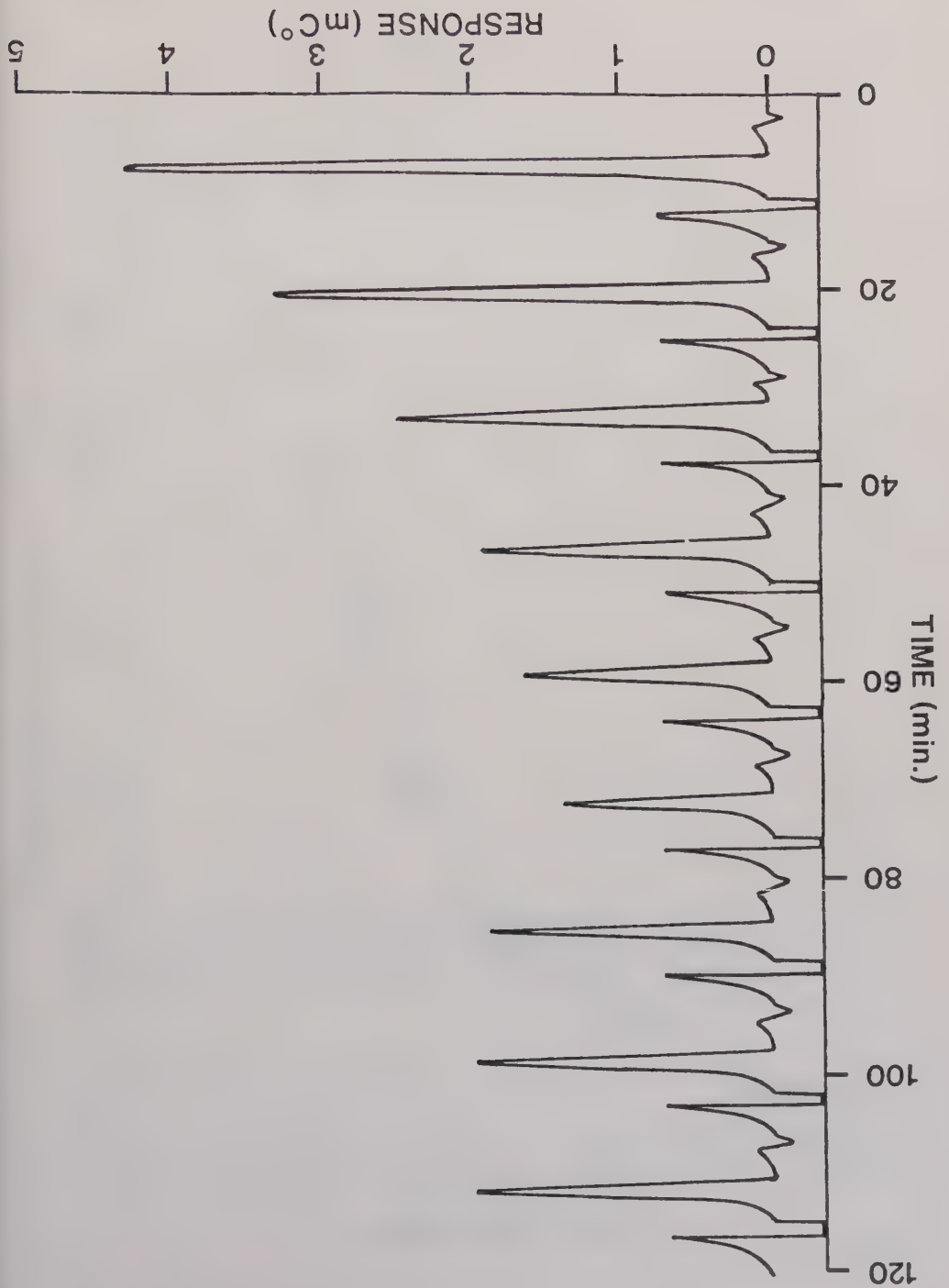
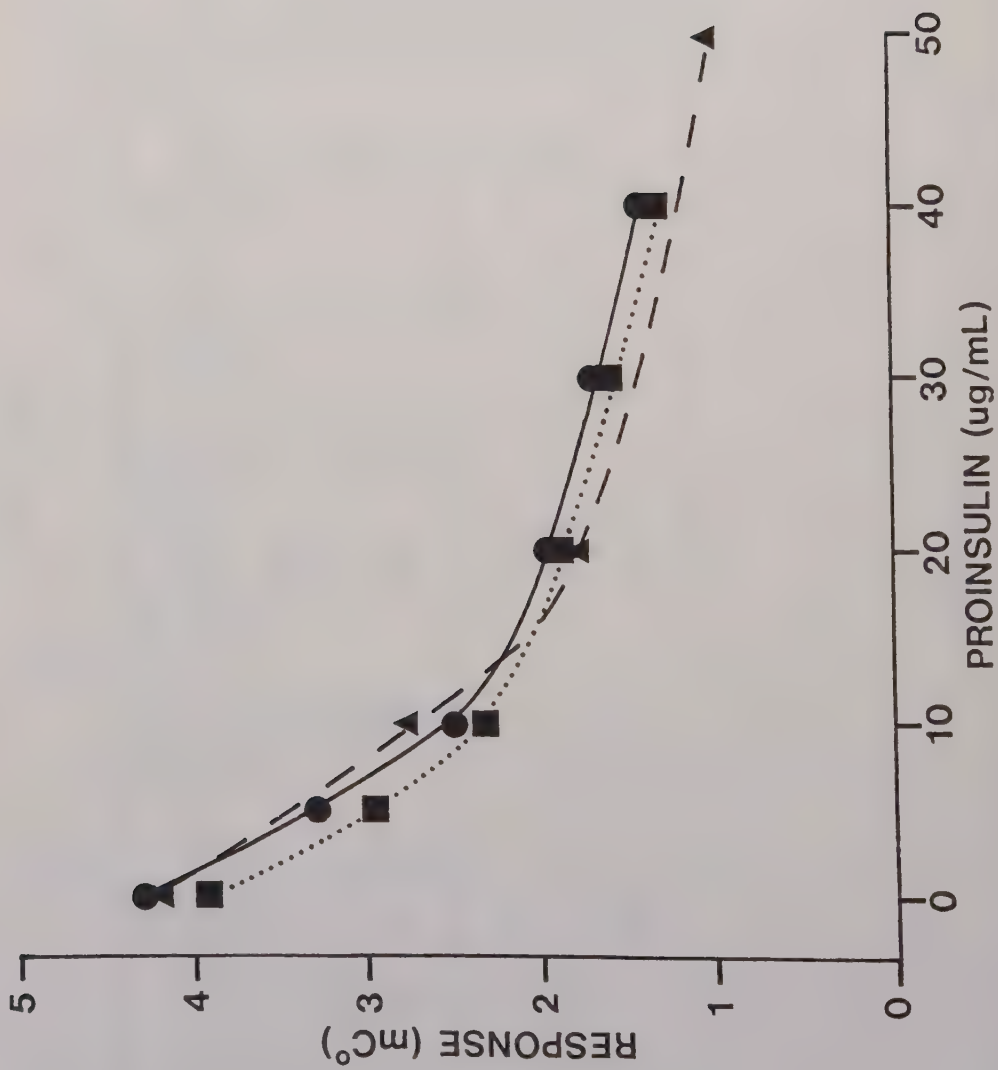


Fig. 6



"The Chimaera was held to be unconquerable. She was a most singular portent, a thing of immortal make, not human, lion fronted and snake behind, a goat in the middle, and snorting out the breath of the terrible flame of bright fire".

Homer, The Iliad

PREPARATION OF A SOLUBLE BIFUNCTIONAL ENZYME BY GENE FUSION

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Within the cellular framework are a number of sequentially operating enzyme systems, in many cases in the form of bi- or even multifunctional proteins each consisting of a single type of polypeptide chain but having two or more catalytic or binding functions. In order to study the properties of an artificial bifunctional enzyme, an in-frame fusion of the structural

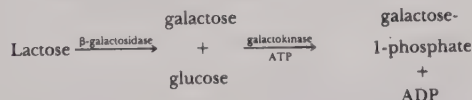
genes for β -galactosidase (*lacZ*) and galactokinase (*galK*) of *Escherichia coli* was made *in vitro*. This DNA chimera was constructed by connecting the 3'-terminus of the *lacZ* gene to the 5'-terminus of the *galK* gene. The translated gene product was able to catalyze the sequential reaction normally carried out by the separate native enzymes.

The modern conception of cellular metabolism pictures a highly branched and complex metabolic pathway. Molecular activities within the cell are finely tuned and complex interlocking regulatory circuits provide a proper flow of substrates and products optimal for cell maintenance. In order to gain insights into these aspects of metabolic regulation, and more specifically to analyze the effects of proximity on the overall turnover of sequentially operating enzyme systems or of coenzyme recycling enzyme systems, we^{1,2} and other laboratories³ have in the past immobilized various enzymes either to polymer matrices such as agarose or by chemical crosslinking⁴. More recently, we have described a two-enzyme system with the enzymes arranged in an oriented fashion by their crosslinking in the presence of bis-NAD leading to preparations with the active sites of the enzymes juxtaposed to one another⁵. In many of these cases the proximal arrangements of enzymes have led to increased efficiency of the systems studied.

Another approach would be to fuse two enzymes by ligating their structural genes using recombinant DNA techniques. Thus, the naturally occurring stop signals at the 3'-end of one gene and the promoter region at the 5'-terminus of the other gene are removed and upon fusion create a DNA chimera encoding a hybrid protein with two active sites. Previously, Yourno et al.⁶ constructed a bifunctional enzyme by removing a stop signal in the histidine operon of *Salmonella typhimurium* using "classical" bacterial genetics. However, with the development of genetic engineering it has become possible to fuse two distant genes whose products act sequentially.

We decided to choose two well characterized genes with known nucleotide sequences whose products are easily detectable: the structural genes for β -galactosidase (*lacZ*)⁷ and galactokinase (*galK*)⁸ of *E. coli*. *LacZ* gene fusions have been used extensively to study gene expression and gene regulation^{9,10}. The *galK* gene has also been used for gene

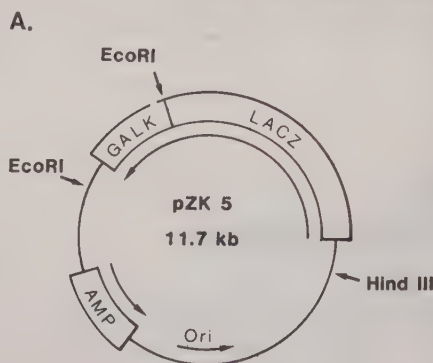
fusion purposes, to study promoter and terminator signals in *E. coli*¹¹. β -galactosidase and galactokinase catalyze the following sequential reaction:



This enzymic conversion of lactose to galactose-1-phosphate represents the first steps in lactose metabolism. The individual enzymic reactions can be monitored with simple and sensitive assays^{12,13}.

The plasmid constructed for these experiments was pZK5. This plasmid was prepared by fusing a 1350-base pair DNA fragment encoding galactokinase to the 3'-terminus of *lacZ* (Fig. 1). The DNA chimera encodes the first 1007 amino acids of β -galactosidase followed by 378 amino acids of galactokinase. The hybrid protein thus lacks 16 carboxyl-terminal residues¹⁴ of β -galactosidase and four amino-terminal residues of galactokinase. When transformed to the host strain *E. coli* C600 (*galK*⁻) (provided by K. McKenney) or *E. coli* DG 75 (*lacZ*⁻)¹⁵ the fusion protein comprises 3 percent and 2 percent of total protein, respectively.

We were able to purify the hybrid enzyme using only a few and very simple steps: 1 and 5M urea treatments of the cell debris followed by batch chromatography on DEAE-Sephadex (Table 1). When the cells were harvested and lysed by sonication most of the β -galactosidase/galactokinase activities (70 percent) were found together with the cell debris. This solid material was treated with 1 M urea. This step became necessary since the hybrid protein tended to form insoluble aggregates with contaminating proteins if it was omitted. The β -galactosidase/galactokinase activities could thereafter be extracted by 5 M urea. At this stage the protein was tolerably pure but ATPase



B.

1	2	3	4	5	6
ATG	AGT	CTG	AAA	GAA	AAA
a. MET SER LEU LYS GLU LYS-					
1005	1006	1007	1008	1009	1010
GCG	GAA	TTC	CAG	CTG	AGC
b. -ALA GLU PHE GLN LEU SER-					
1005	1006	1007		5	6
GCG	GAA	TTC	CAA	GAA	AAA
c. -ALA GLU PHE GLN GLU LYS-					

FIGURE 1 A. Construction of pZK5. A 1350-base pair DNA fragment encoding galactokinase was isolated from YRpR117 by digestion with EcoRI. As source of the *lacZ* gene λ plac5 DNA¹⁵ was used. The gene was cloned as a Hind III/EcoRI fragment in pBR322. The resulting plasmid (pz12) contains a unique EcoRI site in the 3'-end of *lacZ*. pZK5 was obtained by digestion of pz12 with EcoRI followed by mixing and ligation with the *galk* fragment. Recombinants containing a functional *lacZ* gene were scored by plating on Xgal plates¹⁵. Galactokinase positive transformants were detected as red bacterial colonies on McConkey galactose plates¹¹. B. Predicted amino acid sequence from the nucleotide sequence. a. Amino-terminus of native galactokinase. b. Part of carboxyl-terminus of native β -galactosidase. c. Junction region of the hybrid protein.

activity necessitated another purification step. The hydrophobic nature of the bifunctional enzyme precluded use of normal column chromatographic procedures. However, absorption to DEAE-Sephadex in batch proved to be very useful and resulted in a protein with at least 80–90 percent purity, as judged from SDS/PAGE analysis. Figure 2 presents the results of electrophoretic analyses of the purification steps. The mobility of the hybrid protein band indicates a molecular weight (MW) for the protein of 151,000 for each subunit. This correlates well with the sum of the molecular weights of β -galactosidase monomers (MW 116,000) and galactokinase (MW 40,000). The amino acids from the native enzymes removed in the gene fusion have a molecular weight of approximately 2,500.

The electrophoretic properties of the fusion protein

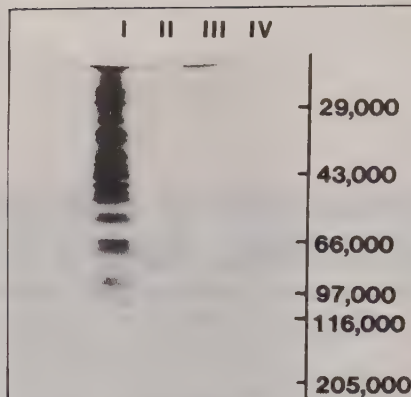


FIGURE 2 Sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis of the hybrid protein. The samples were subjected to SDS-gel electrophoresis on 10 percent polyacrylamide gels. The gel was stained with Coomassie brilliant blue. Molecular weight standards, electrophoresed simultaneously, were myosin (205,000), β -galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (43,000) and carbonic anhydrase (29,000). I. After sonication; II. 1 M urea; III. 5 M urea; IV. After DEAE-Sephadex treatment.

were also analyzed under nondenaturing conditions. When electrophoresed in a 0.7 percent agarose gel (40 mM Tris-acetate, pH 8.0, 2 mM EDTA) the fusion protein could be separated from purified native β -galactosidase and galactokinase. β -galactosidase activity was detected by the fluorogenic substrate 4-methylumbelliferyl- β -D-galactopyranoside¹⁵ while galactokinase activity was determined by dissection of the gel followed by incubation with ¹⁴C-galactose and ATP¹². The fusion protein showed an electrophoretic mobility between that of native β -galactosidase and galactokinase. However, the two enzymic activities of the hybrid enzyme were found superimposed. Determination of isoelectric points (pI) gave similar results: β -galactosidase, galactokinase and fusion protein have pIs of 5.1, 5.8 and 5.5, respectively.

To test the stability against thermal denaturation, the purified bifunctional enzyme was heated to 40°C in 40 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1.0 mM MgCl₂ and 5 mM β -mercaptoethanol at protein concentrations of 0.2–0.3 mg/ml. After 30 minutes only 10 percent of the galactokinase activity remained while the β -galactosidase part remained stable. The native enzymes were completely stable at this temperature. After 30 minutes at 50°C 8 percent of the activity of the β -galactosidase part of the hybrid remained while the galactokinase was totally inactivated. The native β -galactosidase and galactokinase had 100 and 20 percent remaining activity, respectively. The hybrid enzyme is also more sensitive to denaturation by urea treatment and freezing than the separate enzymes. Taken together, these results indicate that the hybrid enzyme, although active, has a slightly perturbed structure.

The galactokinase moiety of the bifunctional enzyme showed a specific activity corresponding to 50–70 percent of the native enzyme. However, galactokinase is a monomeric enzyme while β -galactosidase is tetrameric implying that the important role of subunit interactions must be born in mind. (We assume the configuration of the bifunctional enzyme is tetrameric, with each β -galacto-

TABLE 1 Purification of β -galactosidase/galactokinase.

Fraction	Volume (ml)	Total protein (mg)	β -Galactosidase			Galactokinase		
			Total activity (U)	Specific activity (U/mg)	Recovery (%)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
1. After sonication	1,000	10,500	44	0.0042	100	600	0.057	100
2. 5 M urea	14	36	28	0.78	64	288	8.0	48
3. DEAE-Sephadex	13	3.9	5.3	1.36	12	54	13.8	9

The hybrid protein was purified from *E. coli* DG75 carrying pZK5 grown to late exponential phase ($OD_{550}=3$) in 1 liter of LB medium¹⁵ containing 100 mg/l of ampicillin. The cells were harvested and washed once in 100 ml 0.1 M Tris-HCl, pH 7.8, 1 mM $MgCl_2$, 0.5 mM EDTA and 0.1 M NaCl (buffer A). After centrifugation, the wet cell paste was suspended in 30 ml of buffer A. Thereafter, cells were lysed by sonication (10 $\times$ 15 seconds, Sonifier B-30, Branson Sonic Power) and the cell debris was pelleted by centrifugation at 48,000 $\times$ g for 15 minutes. The pellet was dissolved in 10 ml of 1 M urea and centrifuged at 48,000 $\times$ g for 30 minutes. The supernatant was dialyzed overnight against 2 l of buffer A. The dialysate was absorbed to an equal volume of DEAE-Sephadex A-25 (previously equilibrated in buffer A) and the suspension was mixed end over end for 1 hour at 4°C. The hybrid protein could be eluted with buffer A containing 0.2 M of NaCl. β -galactosidase was assayed by a colorimetric assay using o-nitrophenyl- β -D-galactoside (ONPG) as substrate¹⁵. The assay was performed at 20°C and the activity was measured at 420 nm. One unit represents a change of one A unit/min. at 420 nm. Galactokinase activity was determined using ¹⁴C-galactose¹². One unit of enzyme phosphorylates 1 nmole of galactose/minute at 30°C. Protein concentrations were determined by the Bradford method¹⁶.

sidase monomer carrying a galactokinase unit.) The relatively low obtained specific activity of β -galactosidase (Table 1) could be accounted by the fact that the COOH-terminal amino acids of the native enzyme are involved in monomer-monomer interactions¹⁶. The normal subunit interactions of the hybrid protein are thus likely partially impeded. It remains to be tested if a higher activity can be obtained if galactokinase is fused to the amino-terminus of β -galactosidase.

The purified hybrid enzyme was able to catalyze the hydrolysis of lactose and the subsequent phosphorylation of galactose. The ADP formed in the total enzymic reaction was measured spectrophotometrically at 340 nm with phosphoenolpyruvate, pyruvate kinase (PK), lactate dehydrogenase (LDH) and NADH by the rate of oxidation of NADH¹⁹. The assay showed a complete requirement for lactose, ATP, phosphoenolpyruvate, LDH, PK and hybrid enzyme.

In our forthcoming work, we intend to study the proximity effects of bifunctional enzymes alone, or in the presence of competing enzymes, to evaluate the possibilities of substrate channeling.

Constructions such as the hybrid enzyme described here allow simultaneous regulation of two structural genes; one of the suggested motives behind the evolution of bifunctional enzymes²⁰. The preparation of bifunctional enzymes using gene fusion should be of practical and theoretical interest in many cases both *in vivo* and *in vitro*. Thus, we are currently preparing antigen-enzyme conjugates for ELISA purposes by gene fusion. Protein purification as well as protein immobilization should be considerably simplified since both enzymic activities are purified and handled at the same time. Systems for coenzyme regeneration and coupled enzymic systems, e.g. spectrophotometric analyses, should be important fields of application. In addition, the technique represents an important tool in studies of subunit interactions.

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Characterization of an artificial bifunctional enzyme, beta-galactosidase/galactokinase, prepared by gene fusion

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Enzymes and Abbreviations

Enzymes: galactose dehydrogenase (EC 1.1.1.48); galactokinase (EC 2.7.1.6); restriction enzymes EcoRI, BamHI, SalI, PstI, HindIII (EC 3.1.24.4); S1 nuclease (EC 3.1.30.1); beta-galactosidase (EC 3.2.1.23); T4 DNA ligase (EC 6.5.1.1)

Abbreviations: SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; bp, base pair; Xgal, 5-bromo-4-chloro-3-indolyl-beta-D-galactoside; IPTG, isopropyl-thio-beta-D-galactoside; ONPG, 2-nitrophenyl-beta-D-galactopyranoside; DTT, dithiothreitol

Summary

An artificial bifunctional enzyme, beta-galactosidase/galactokinase, has been prepared by gene fusion. The hybrid protein catalyzes the hydrolysis of lactose followed by phosphorylation of galactose. The protein has been purified using DEAE-Sephacel chromatography and gel filtration on Sephacryl S-400 Superfine. The configuration of the hybrid protein is mainly tetrameric but also higher aggregates can be detected. The monomer M_r is 160,000 as judged from sodium dodecyl sulfate/polyacrylamide gel electrophoresis and the native M_r has been calculated to be 600,000-650,000 from gel filtration experiments. Beta-galactosidase/galactokinase has different thermostability curves, pH activity profiles and K_m values as compared with the native enzymes. By using a third enzyme, galactose dehydrogenase, which competes with galactokinase for the galactose formed by beta-galactosidase, substrate channeling can be detected. This proximity effect becomes even more pronounced in an assay mixture containing poly(ethylene glycol).

Introduction

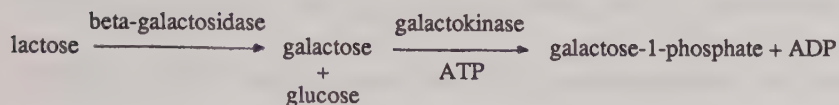
Cell metabolism is characterized by the action of enzyme sequences. From the emerging views on enzymic infrastructure it is clear that there is a spatial organization of enzymes in a metabolic sequence. The modes of such an organization entail a wide scope of protein complexes from the weak protein-protein interactions found between e.g. the glycolytic enzymes (1) to the multifunctional proteins possessing more than one autonomous catalytic or binding site on the same polypeptide chain.

A wide variety of techniques, including immobilization of sequentially acting enzymes to a solid matrix (2,3), crosslinking of enzymes in a random fashion (4) or in an oriented fashion with their active sites juxtaposed (5), have been applied to gain a better understanding into these proximity effects. In many of these cases, the organized enzyme systems exhibited different forms of kinetic or catalytic facilitation, compared to their counterpart systems free in bulk solution.

An attractive alternative approach to the above described systems would be to fuse two enzymes by ligating their structural genes using recombinant DNA-techniques. This procedure mimics the evolution of naturally occurring bifunctional enzymes which most probably have evolved from smaller proteins through gene fusion (6). In a recent paper

(7) an approach to prepare a bifunctional beta-galactosidase/galactokinase was described.

The complex could catalyze the following sequential reactions:



In this study an improved beta-galactosidase/galactokinase hybrid protein was prepared.

This artificial bifunctional enzyme was isolated from E. coli carrying plasmid pZK 105

encoding an in frame fusion between the first 1021 (out of 1023) residues of beta-

galactosidase followed by a 20 residues long linker region and 378 (out of 382) carboxyl-

terminal residues of galactokinase. In order to elucidate the microenvironmental effects

caused by the gene fusion various functional features of the beta-

galactosidase/galactokinase protein such as substrate channeling, stability and pH-

optimum were examined. These investigations should give a clue to what selective

advantage covalently linked proteins might have had during evolution as well as to give a

better understanding to the applied uses of enzyme sequences in enzyme technology.

MATERIALS AND METHODS

Chemicals and reagents

Restriction enzymes, S1 nuclease, T4 DNA ligase and beta-galactosidase were purchased from Boehringer Mannheim. Galactose dehydrogenase, isopropyl-thio-beta-D-galactoside (IPTG), 2-nitrophenyl-beta-D-galactopyranoside (ONPG) and 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (Xgal) were from Sigma. [$1-^{14}\text{C}$]galactose and [$\alpha-^{35}\text{S}$]dATP were purchased from Amersham and New England Nuclear, respectively. Acrylamide, bisacrylamide and sodium dodecyl sulfate (SDS) were obtained from Biorad. Poly(ethylene glycol) 4000 was from Merck. Sephacryl S-400 Superfine, DEAE-Sephacel and a kit containing high molecular weight standard proteins for gel filtration were from Pharmacia Fine Chemicals. All other reagents were commercially available and of analytical grade.

Bacterial strains, plasmids and indicator plates

E.coli strain F'11 rec A((lac, pro) Δ thi, rif^A, str^A, recA/F'lacIqZ⁻, pro⁺) (8) which overproduces lac repressor 100-fold and E.coli strain C 600K⁻ (galE⁺T⁺K⁻, lac⁻, thr⁻, leu⁻), obtained from K. McKenney, were used for standard transformation procedures. Plasmids pUR 292 (9) and pZK 5 (7) were described earlier. LacZ⁺ colonies were identified on indicator plates containing Xgal, a

chromogenic substrate of beta-galactosidase, the inducer IPTG and ampicillin (10). A blue colour of colony indicates the presence of beta-galactosidase activity. Galactokinase positive transformants were detected as red bacterial colonies on McConkey galactose plates (11).

Construction of plasmids

Restriction enzyme digests and other cloning procedures were performed as described (12).

Plasmids were prepared according to Birnboim and Doly (13). The nucleotide sequence of the linker region were determined by the Sanger dideoxy chain termination method (14) using the modifications of Messing (15) except that [^{35}S]dATP was used as radioactive nucleotide.

Purification of beta-galactosidase/galactokinase

The artificial beta-galactosidase/galactokinase was purified according to a protocol by Fowler and Zabin (16) with some minor modifications. The hybrid protein was isolated from *E.coli* F'11 recA carrying pZK 105 grown to late exponential phase in LB-broth (10) supplemented with 50 mg/ml of ampicillin and 0.2 mM IPTG. The cells were spun down at 10,000 x g for 10 min and washed once with 0.04 M Tris-HCl pH 8.0 containing 0.5 mM EDTA, 1 mM MgCl_2 and 1 mM DTT (buffer A). All subsequent operations were carried out at 0-4 °C. After resuspension in the same buffer bacterial extracts were prepared by sonic disintegration and were clarified by

centrifugation at 20,000 x g for 30 min. Solid ammonium sulfate was then added slowly to the supernatant until 40 % saturation was reached. After removing the precipitate by centrifugation at 20,000 x g for 30 min, it was dissolved in buffer A and dialyzed overnight against buffer A. Any precipitate was removed by centrifugation at 40,000 x g for 10 min. The supernatant of the preceding step was applied to a column of DEAE-Sephacel previously equilibrated with the same buffer. After washing with 10-15 column volumes, a linear gradient of 0.1-0.4 M NaCl was applied. Fractions with both beta-galactosidase and galactokinase activities were combined and concentrated by ammonium sulfate precipitation. The protein was dissolved in a minimum volume of 0.1 M Tris-HCl containing 1 mM MgCl₂, 0.5 mM EDTA and 1 mM DTT. The protein solution was finally subjected to gel filtration on a column of Sephacryl S-400 Superfine.

Enzyme assays

Beta-galactosidase was assayed by hydrolysis of ONPG (10). One unit of enzyme hydrolyzes 1 μ mole of ONPG, corresponding to 0.05 μ mole lactose, per minute at 30 °C. Galactokinase activity was determined using [¹⁴C]galactose (11). One unit of galactokinase phosphorylates 1 μ mole of galactose per minute at 30 °C. 0.1 M Tris-HCl pH 8.0 containing 10 mM MgCl₂ and 10 mM mercaptoethanol was used as buffer.

In the "scavenger" enzyme assay with galactose dehydrogenase 10 μ l of bifunctional enzyme solution, i.e. 0.007 U beta-galactosidase (measured with lactose as substrate) and 0.01 U galactokinase, or 10 μ l solution of separate native enzymes with the same enzyme activities was added to 990 μ l 0.1 M Tris-HCl pH 8.0 containing 10 mM lactose, 10 mM ATP, 10 mM $MgCl_2$, 10 mM mercaptoethanol and 0.01 U galactose dehydrogenase. In order to monitor the competition between galactokinase and galactose dehydrogenase for the galactose formed by the hydrolysis of lactose, NAD was added to a final concentration of 1 mM. The amount of NADH was measured fluorimetrically at 360 nm (band width 5 nm) and 460 nm (band width 5 nm) excitation and emission wavelengths, respectively. The fluorescence measurements were carried out with a Hitachi fluorescence spectrophotometer, type U-3200.

Protein determination

Protein was determined by the method of Bradford (17) using bovine serum albumin as standard.

Electrophoresis in polyacrylamide gel

SDS-PAGE was performed on 8 % polyacrylamide slab gels using a Tris-glycine pH 8.3, discontinuous buffer system as described by Laemmli (18). The M_r of the enzyme subunits were determined by comparing their relative mobilities with those of the standard proteins: myosin (M_r

= 205,000); beta-galactosidase (116,000); phosphorylase b (97,000) bovine serum albumin (66,000) and ovalbumin (43,000).

Heat stability measurements

Heat stability measurements of bifunctional enzyme, beta-galactosidase and galactokinase were carried out at 45 °C and 54 °C in 0.1 M Tris-HCl pH 8.0 containing 1 mM MgCl₂, 1 mM DTT and bovine serum albumin to a final protein concentration of 1 mg/ml.

Results

Construction of pZK 105

The strategy used for the construction of a plasmid with an in frame fusion between the structural genes of beta-galactosidase (lacZ) and galactokinase (galK) is outlined in figure 1. pZK 5 (7) encodes a bifunctional beta-galactosidase/galactokinase lacking 16 carboxyl-terminal amino acids of beta-galactosidase. Initially, one of the two EcoRI sites of pZK 5 was removed by digesting the plasmid partially with EcoRI followed by S1 nuclease treatment. The obtained plasmid pZK 19 simplified the following screening procedures substantially.

A 120 bp DNA fragment encoding the missing amino acids of beta-galactosidase and a linker region was obtained by digesting pUR 292 with EcoRI. This fragment was inserted in the EcoRI site of pZK 19. Use of E. coli C600K⁻ as host cells resulted in gal⁻ transformants due to an incorrect translational reading frame of pZK 47. When transformed into E. coli F'11recA selection on indicator plates yielded lac⁺ colonies which were screened for plasmids with a size of 11,700 bp. Plasmid pZK 105 was isolated by digesting pZK 47 partially with EcoRI followed by treatment with S1 nuclease. Ligation and transformation into E. coli C600K⁻ yielded gal⁺ transformants. The reading frame of the fused lacZ and galK genes is in phase encoding a polypeptide of

1,420 residues (figure 2). The linker between the two structural genes codes for 20 residues and contains a polylinker region carrying several restriction enzyme sites.

Purification of beta-galactosidase/galactokinase

The hybrid protein could be purified using a protocol originally developed for beta-galactosidase. A summary of the purification steps is given in Table 1. Two chromatographic steps, ion exchange chromatography on DEAE-Sephacel (figure 3) and gel filtration on Sephacryl S-400 Superfine (figure 4) were employed. The elution profiles indicated that the two activities copurified. Besides the galactokinase activity from the bifunctional enzyme, *E. coli* F'11 recA also expressed a chromosomal galK gene. However, this native galactokinase was readily separated from the bifunctional enzyme in the ammonium sulfate precipitation and ion exchange chromatography steps. As judged from SDS-PAGE the hybrid protein was at least 95 % pure. The bifunctional enzyme had specific activities of 200 U/mg of beta-galactosidase and 14 U/mg of galactokinase. When corrected for the increase of M_r caused by the gene fusion, these specific activities correspond to 80-90 % and 60-70 % of those of wild-type beta-galactosidase and galactokinase, respectively.

It should be noted that on some occasions proteolysis was detected near the site of joining of the two polypeptides as judged by SDS-PAGE. This could be avoided by applying the protein on the ion exchange column without delay.

Molecular weight determination

The molecular weight of the hybrid enzyme was estimated by gel filtration on Sephacryl S-400 Superfine. The protein eluted as two peaks with the main peak corresponding to a molecular weight of 600,000-650,000 and the other peak to 900,000-950,000.

The molecular weight determined by SDS-PAGE was estimated to 160,000. Beta-galactosidase is a tetrameric enzyme with $M_r = 116,000$ for each subunit while galactokinase is a monomeric enzyme with $M_r = 40,000$. Including the linker region, the monomer of the hybrid has a theoretically calculated M_r of 157,000. Thus, these determinations suggests that the bifunctional enzyme has a tetrameric configuration where each beta-galactosidase subunit carries a galactokinase molecule. However, a considerable fraction (10-20 %) of the protein solution consists of active forms of the hybrid containing 6 monomers. This is in agreement with native beta-galactosidase where active aggregates of 6 up to 16 monomers are found (19).

pH optimum

The activity as a function of pH for the bifunctional enzyme and the corresponding native enzyme is shown in fig 5. In 0.1 M Tris-HCl, the pH optimum of beta-galactosidase moiety of the bifunctional enzyme was between 7.9 and 8.1 while that of native beta-galactosidase was between 7.1 and 7.3. The pH optima of the galactokinases, native and bifunctional, were coinciding at 8.3-8.5.

Thermostability

To test the stability of the native and the hybrid proteins against thermal denaturation, the enzymes were heated to 45 °C and 54 °C, respectively. As can be seen from figure 6, the modified enzyme was slightly more sensitive to high temperatures, especially above 50 °C.

Determination of K_m values

The dependence of the initial rates on the concentrations of ONPG, galactose and ATP were determined for the native and hybrid enzymes. When plotted according to the method of Lineweaver and Burk these data yielded K_m values as shown in Table 2.

Substrate channeling

In order to investigate the proximity effects and the possibilities of substrate channeling, a third enzyme, galactose dehydrogenase, was introduced in the assay mixture as a competitive enzyme, a probe or a "scavenger" enzyme (20), to galactokinase. Galactose dehydrogenase which oxidases galactose to galactolactone was used in an assay solution consisting of either the bifunctional enzyme or a comparable system of the two native separate enzymes (figure 7). When the assay system described in Materials and Methods was employed, 59 % of the galactose formed by lactose hydrolysis was phosphorylated by galactokinase using the bifunctional enzyme. In a corresponding system with native enzymes 48 % of the galactose was phosphorylated, thus indicating at least a partial substrate channeling. In order to mimic the intracellular milieu the kinetic behaviour of the native and the bifunctional enzyme systems was also studied in media containing increasing concentrations of poly(ethylene glycol). When the fraction of galactose phosphorylated was plotted against poly(ethylene glycol) concentration both systems were found to be influenced by the polymer in the medium (fig. 8). In both cases the amount of galactose phosphorylated increased. However, the increase for the bifunctional

enzyme was considerably higher, yielding 90% galactosidase phosphorylated at 100 g/l poly(ethylene glycol) compared with 68 % for the native system.

Discussion

In this study a bifunctional beta-galactosidase/galactokinase hybrid protein has been prepared by gene fusion. The purified artificial enzyme is able to catalyze the hydrolysis of lactose and the subsequent phosphorylation of galactose, thus indicating that the polypeptide chain can fold sufficiently well to yield autonomous domains. However, the novel microenvironment created in the gene fusion event presents new physical and kinetic properties with the hybrid.

Physical properties of the hybrid protein

The pH optimum for beta-galactosidase of the bifunctional enzyme is shifted by 0.8 pH units to the alkaline side. A number of papers, e.g. (21,22), have reported that the pH activity profiles of several enzymes when adsorbed to a negatively charged matrix are displaced towards more alkaline pH values by 1-2.5 pH units as compared with the free enzymes. This charge effect is considered to be due to the microenvironment of the adsorbed enzyme becoming more acidic than that of the bulk solution since the negatively charged surroundings attract protons thereby reducing the local pH value. A similar explanation of the observed pH effect of the bifunctional enzyme could be given. Beta-galactosidase might be looked upon as "immobilized" to a negatively charged galactokinase molecule, since pI of galactokinase is 5.8 (7). Thus, the

micromilieu surrounding the beta-galactosidase moiety might be considerably changed.

However, it cannot be excluded that the gene fusion disrupts the tertiary structure of beta-galactosidase and the new structure per se generates a different pH optimum.

The K_m values for the bifunctional enzyme as compared with the native enzymes are not changed except for ATP. A possible explanation for the increase in K_m for the bifunctional enzyme can once again be found in a charge effect, in this case in the form of a barrier caused by the beta-galactosidase moiety which is negatively charged ($pI = 5.1$, (7)). In addition, the binding between the beta-galactosidase part and ONPG as well as between the galactokinase part and galactose are not affected.

The bifunctional enzyme exhibits a good thermostability. However, both enzymic activities in the complex are slightly more sensitive to heat denaturation than their native counterparts thus indicating a somewhat perturbed structure. In the case of beta-galactosidase, earlier investigations have shown that hybrid beta-galactosidases exhibit impaired subunit interactions (16). The smallest active form of beta-galactosidase is the tetramer and a loss of activity is mainly due to a separation of the monomers.

Linker region

A number of prokaryotic and eukaryotic genes encoding multifunctional proteins have been sequenced (23,24) and provide insights into the role of regions that connect the catalytic entities. In these multifunctional proteins, comparisons with homologous prokaryotic genes have identified the connecting regions. Most probably, these regions reflect the remnants of ancestral nucleotide sequences involved in the genetic transposition and fusion of monofunctional genes to create multifunctional genes since there is no or very little sequence homology between different bifunctional enzymes in the linker region as opposed to the functional domains.

Nevertheless, in a bifunctional enzyme designed by in vitro experiments this linker region might help to allow sufficient conformational flexibility to permit both of a correct folding of the domain as well as of optimal subunit interactions. The length of one folded subunit of beta-galactosidase has been estimated to 82 Å in cross section (25). The linker peptide is 20 residues corresponding to a length of 67 Å, if extended, and should thus, theoretically allow for a sufficient extension between the functional domains. However, preliminary investigations have shown that even a hybrid enzyme without a linker region is capable of forming functional domains (7,26). In forthcoming work the intention is to introduce different linkers between the catalytic entities to evaluate their influence on the stability and catalytic efficiency of the hybrid.

Substrate channeling

When studied alone the bifunctional enzyme has no kinetic advantages over a comparable system with native enzymes even in very diluted systems. However, the results obtained with the scavenger enzyme, galactose dehydrogenase, suggest that there is at least a partial substrate channeling. The observed effect is probably due to the fact that the distance between beta-galactosidase and galactokinase is much shorter than between beta-galactosidase and galactose dehydrogenase. The former route is therefore preferred.

Increasing attention has been given to the fact that the protein solutions in the cell are highly concentrated. The presence of a polymer in the assay mixture, e.g. poly(ethylene glycol), mimics the situation in situ by causing a reduction in free volume available for the solute molecules surrounding the enzyme (exclusion effects). Diffusion restriction might also occur. Under such conditions substrate channeling is more pronounced.

Conclusion

It is now understood that most enzymes in a cell act in highly structured states, some of them in the form of bi- or even polyfunctional enzymes. Continued exploration of artificially prepared bifunctional enzymes will most probably be a vital step to the gradual understanding of regulation of cell metabolism. Furthermore, in a number of applications multienzymic reaction sequences are desirable. The use of bifunctional enzymes should then be very attractive since protein purification as well as protein immobilization are considerably simplified because both activities are handled simultaneously.

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Table 1. Purification of beta-galactosidase/galactokinase from E. coli F'11recA/pZK 105

The results presented are for a purification from 8 g cells. Full details are given in Materials and Methods.

Fraction	Volume (ml)	Total protein (mg)	Beta-galactosidase		Galactokinase	
			Total activity (U)	Spec. activity (U/mg)	Total activity (U)	Spec. activity (U/mg)
Homogenate	54	860	3,900	4.5	525	0.61
Ammonium sulphate 0-40 %	8.0	140	2,500	18	230	1.64
DEAE-Sephacel	5.0	12	1,600	133	120	10.0
Sephacryl S-400	45	6	1,200	200	84	14.0

Table 2. K_m values of beta-galactosidase/galactokinase and native enzymes

	Substrate	Native enzyme	Bifunctional enzyme
Beta-galactosidase	ONPG	$3 \times 10^{-4} \text{ M}$	$2 \times 10^{-4} \text{ M}$
Galactokinase	galactose	$3 \times 10^{-3} \text{ M}$	$3 \times 10^{-3} \text{ M}$
	ATP	$6 \times 10^{-5} \text{ M}$	$1 \times 10^{-3} \text{ M}$

FIGURE LEGENDS

- Figure 1.** Schematic representation of the construction of the chimaeric plasmid PZK 105 coding for an in frame fusion between beta-galactosidase (lacZ) and galactokinase (galK).
- Figure 2.** Sequence of nucleotides of the linker region between the fused beta-galactosidase (lacZ) and galactokinase (galK) genes. The numbers indicate the residue numbers of the beta-galactosidase and galactokinase, respectively.
- Figure 3.** DEAE-Sephacel chromatography of beta-galactosidase/galactokinase after ammonium sulfate precipitation. (Fraction volume: 6 ml).
- Figure 4.** Gel filtration on Sephacryl S-400 Superfine of beta-galactosidase/galactokinase pooled from the DEAE-Sephacel column. (Fraction volume: 5 ml).
- Figure 5.** pH activity profiles of beta-galactosidase/galactokinase compared with native enzymes in 0.1 M Tris-HCl.
(● , native enzyme; ○ , bifunctional enzyme)

Figure 6. Heat stability of beta-galactosidase/galactokinase compared with native enzymes. The protein solutions (1mg/ml) were incubated at 45 °C and 54 °C for the indicated length of time and residual activities were determined.

(□ , native beta-galactosidase; ■ , bifunctional beta-galactosidase;
○ , native galactokinase; ● , bifunctional galactokinase)

Figure 7. Schematic representation of the three enzyme system used for the "scavenger" enzyme assay.

Figure 8. The effect of poly(ethylene glycol) on the amount of galactose phosphorylated in the "scavenger" enzyme assay.

(● , bifunctional enzyme; ○ , native enzymes)

Fig. 1

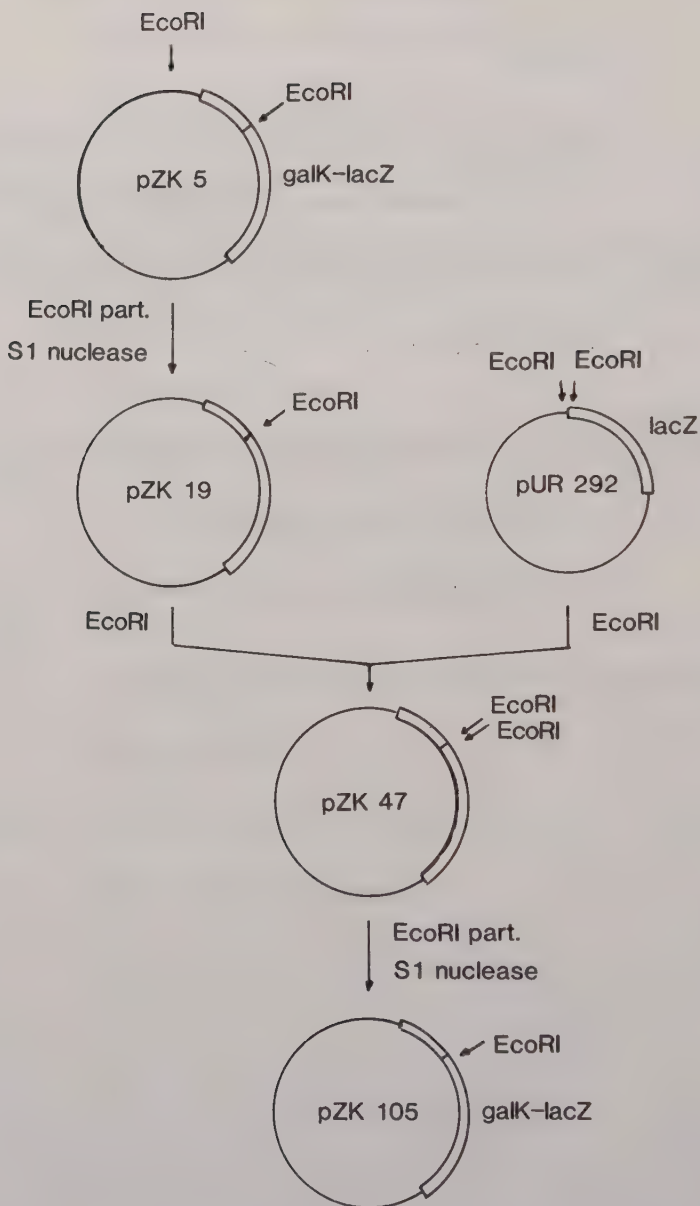


Fig. 2

1020 1021
 lacZ -TGG TGT CAG GGG ATC CGT CGA CCT GCA GCC AAG CTT ATC
 BamHI SalI PstI HindIII
 5 6
 GAT GAT AAG CTG TCA AAC ATG AGC CAA GAA AAA - galK

Fig. 3

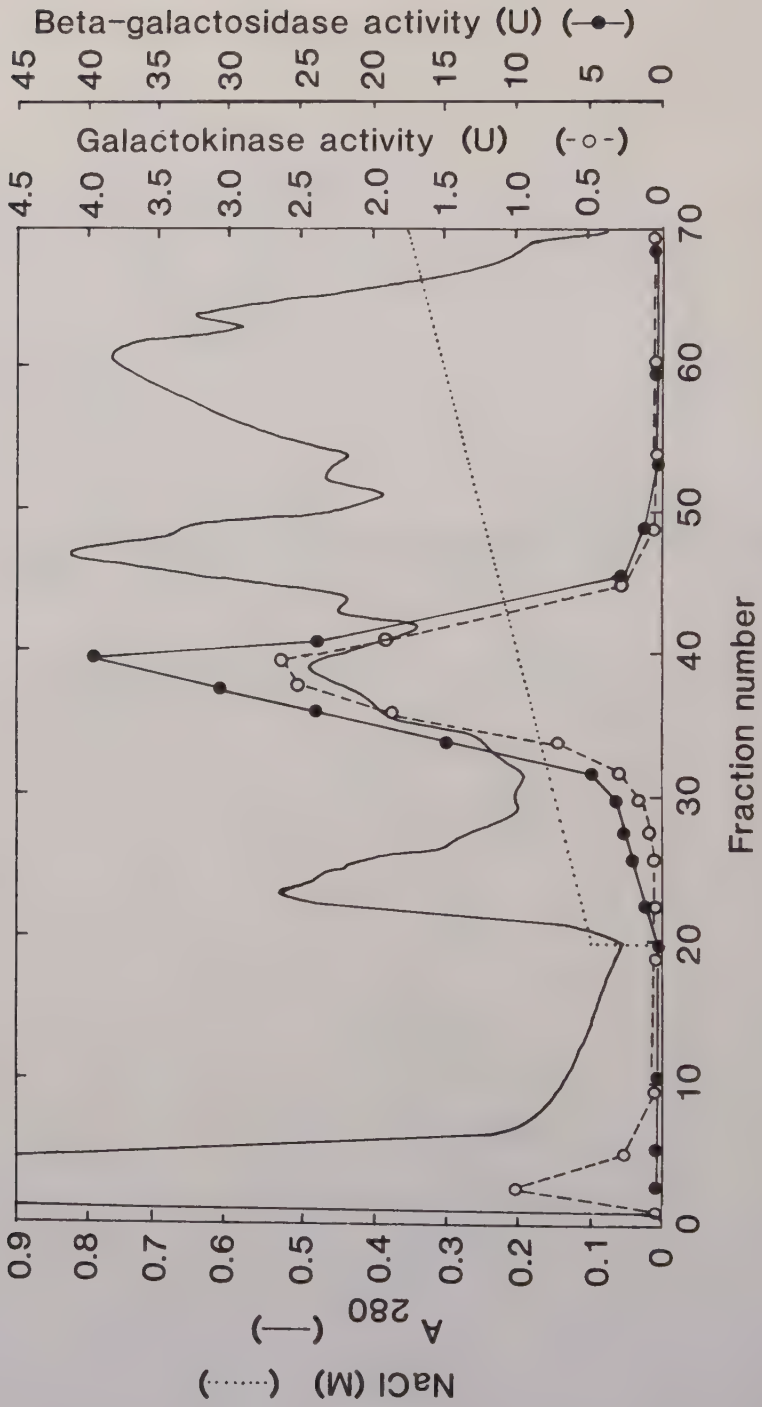


Fig. 4

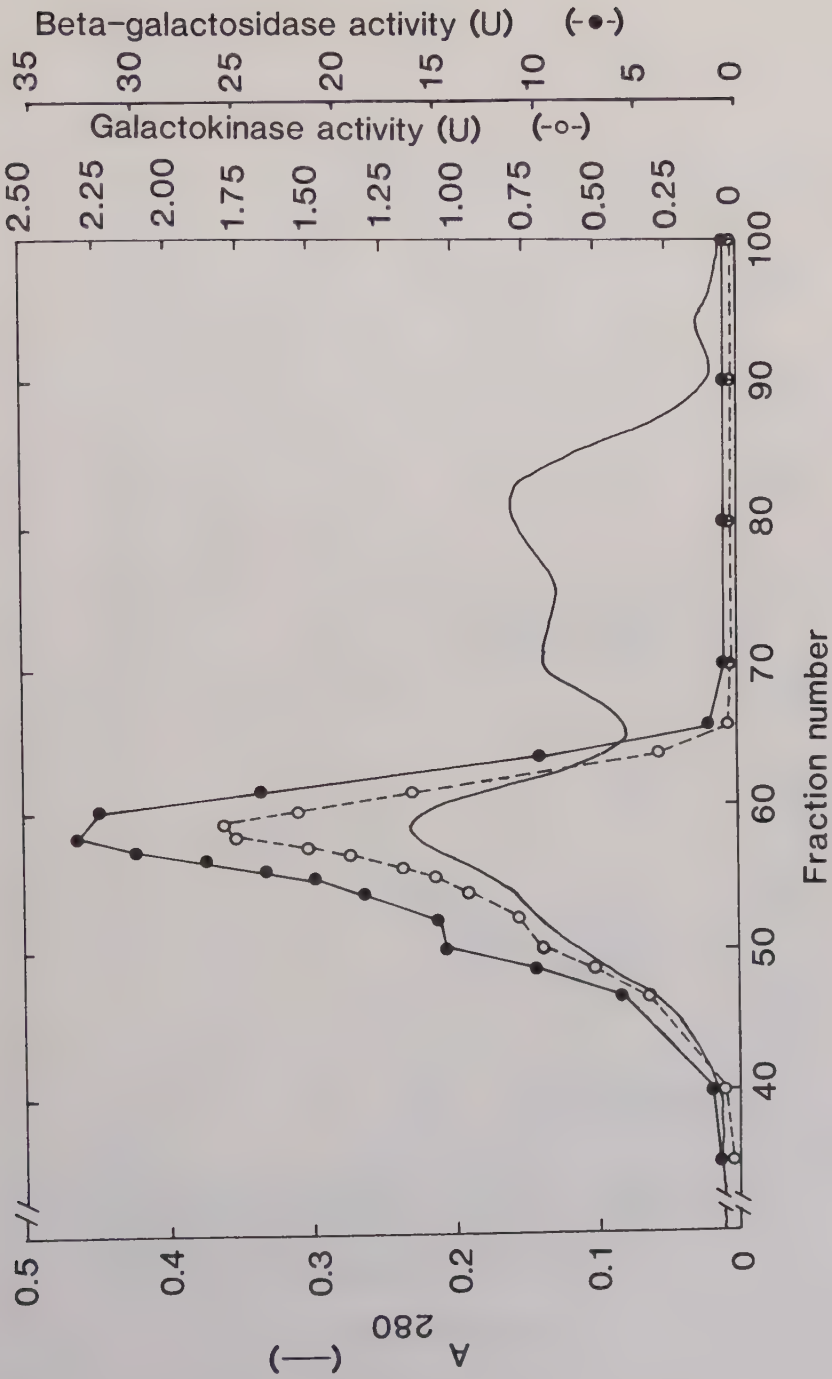


Fig. 5

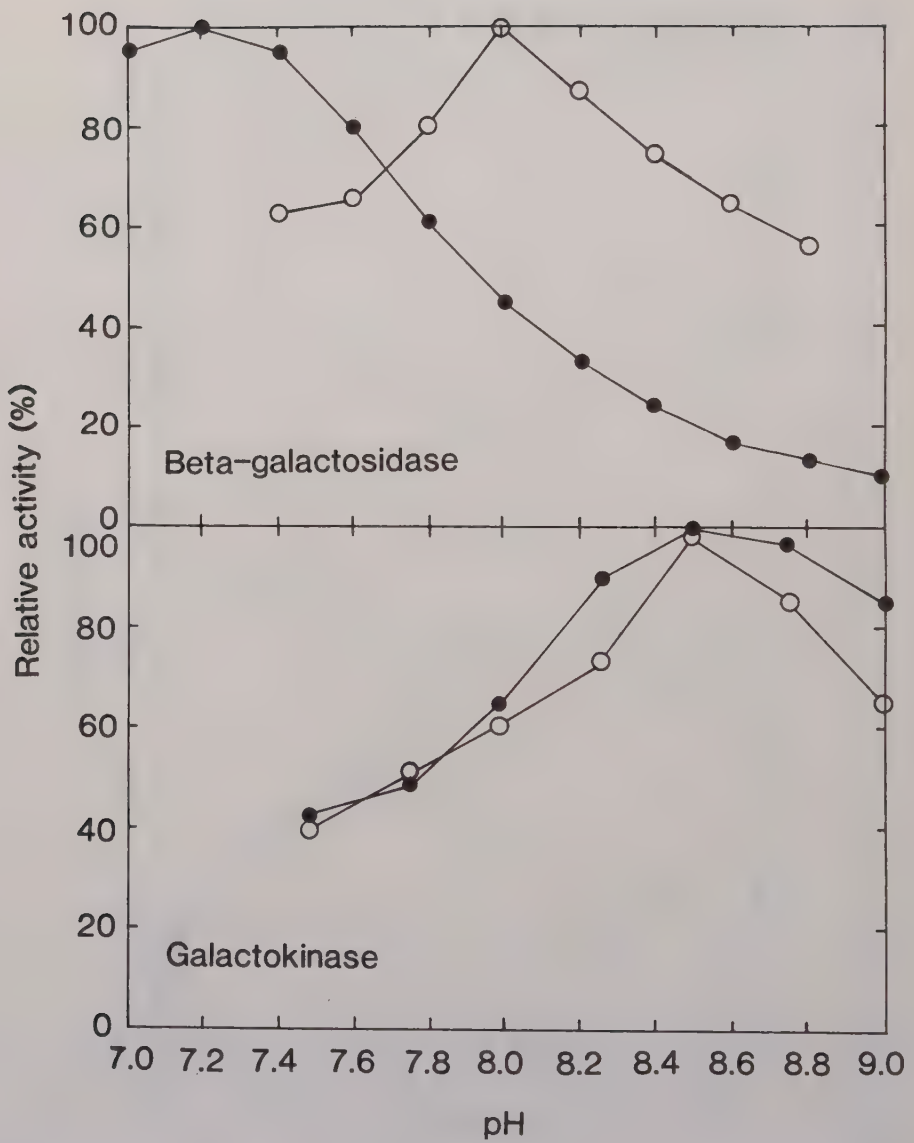


Fig. 6

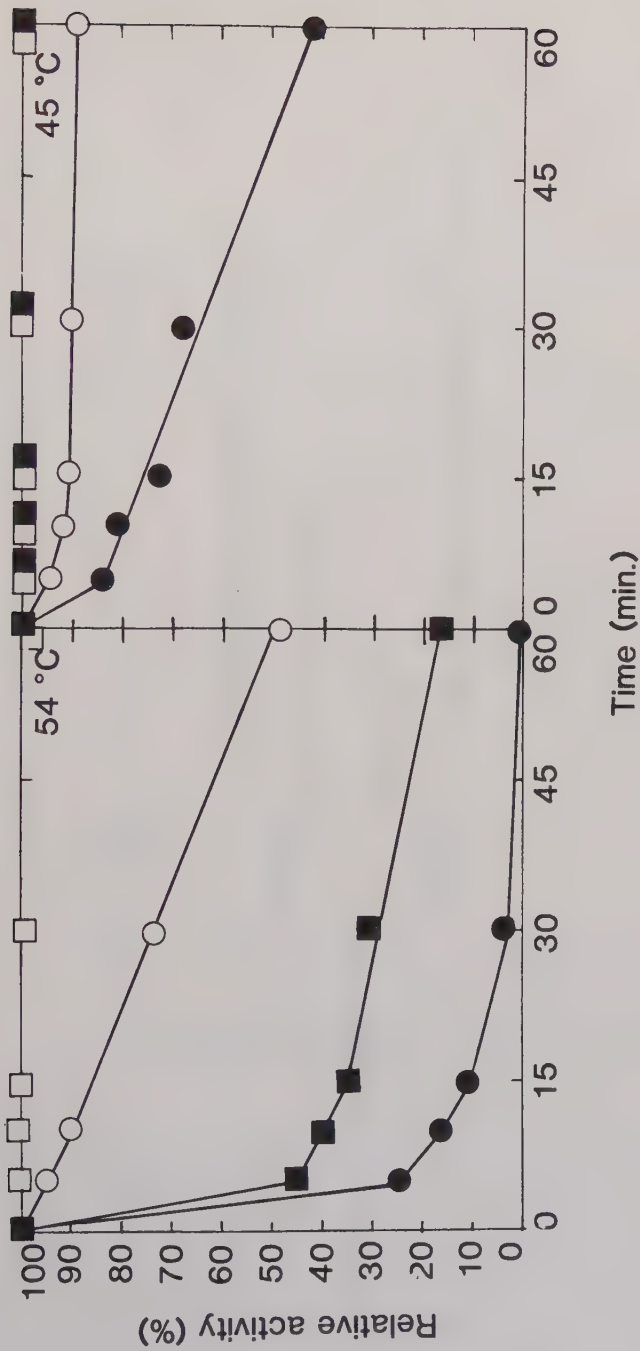


Fig. 7

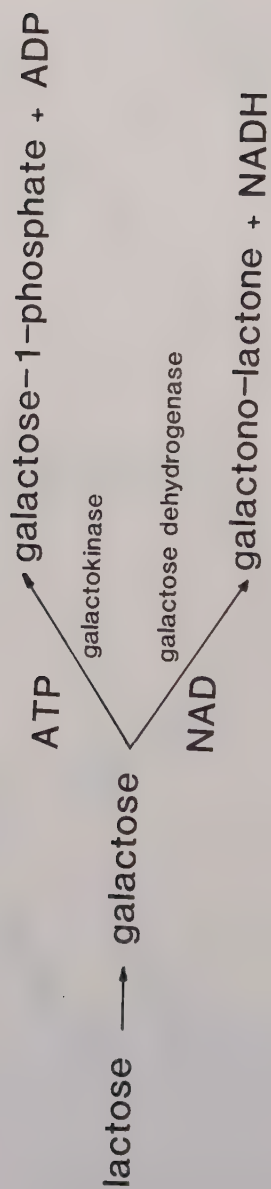
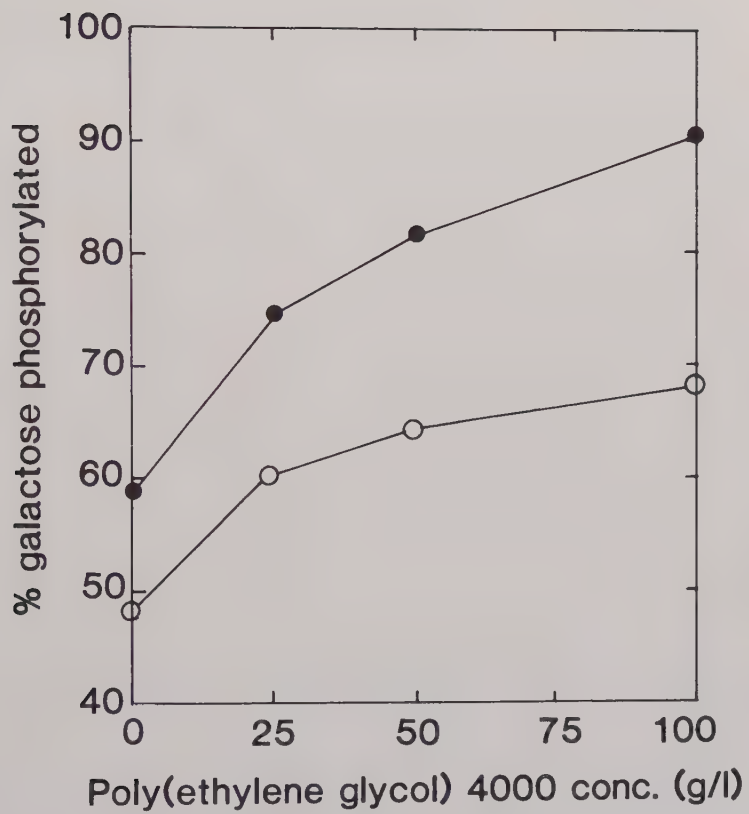


Fig. 8



"When a thing was new people said, It is not true. Later, when the truth became obvious, people said, Anyway, it is not important, and when its importance could not be denied, people said, Anyway, it is not new".

William James

The Expression in E. coli of a Polymeric Gene Coding for an Esterase Mimic Catalyzing the Hydrolysis of p-Nitrophenyl Esters

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ABSTRACT

We have prepared a hybrid protein consisting of seven "esterase" units, glu-ala-his-ala-ser-phe-phe-phe, fused to the N-terminal of galactokinase (E. coli). The structural gene for this bifunctional protein was obtained by cloning a polymer made up of three chemically synthesized oligonucleotides to which the galactokinase gene was fused in frame. The hybrid protein was purified to homogeneity with the aid of the galactokinase moiety and showed a molecular weight of 51,000 - 53,000. The preparation could catalyze the hydrolysis of p-nitrophenyl esters and due to the inbuilt hydrophobic spacers, phe-phe-phe, improved catalysis of more hydrophobic substrates was obtained.

Key words: synthetic esterase/galactokinase/gene fusion/(E.coli)

1. INTRODUCTION

There has been considerable interest over the years to obtain preparations mimicing enzymes, notably of proteases since the rules governing enzyme catalysis are known for many of these enzymes. One line of approach in this direction has been to synthesize by chemical means oligo- and polypeptides consisting of the amino acids believed to participate in hydrolytic reactions (1,2). In more elaborate approaches a "substrate binding pocket" has been built or utilized close or around which the catalytic groups are arranged. Examples of the latter include the use of a solid matrix such as Sephadex (3), the use of cyclodextrins (4,5) or "spherands" (6).

In this study we have applied genetic engineering techniques to examine the possibility of cloning synthetic repeating genes to a peptide expressing hydrolytic activity. We chose to prepare and clone a gene coding for multiple repeats of the octapeptide, glu-ala-his-ala-ser-phe-phe-phe, which contain the catalytic groups necessary for ester hydrolysis. The structural gene of galactokinase (*E. coli*), *galK*, was fused in frame with the synthetic esterase gene. When this chimaeric gene was expressed in *E. coli*, the galactokinase moiety considerably simplified isolation and purification. The obtained hybrid enzyme, esterase-galactokinase, was purified to homogeneity and shown to be hydrolytically active against activated esters. Most likely due to the phe-phe-phe moieties between the catalytic groups, the synthetic esterase showed a higher relative catalytic activity to more hydrophobic substrates as compared with mono-histidine or the histidine containing oligopeptide ser-his-asp.

2. EXPERIMENTAL

2.1. Bacteria and plasmids

E. coli C600 K⁻ (gal E⁺T⁺K⁻, lac⁻, thr⁻, leu⁻), obtained from K. McKenney, was used as a recipient for bacterial transformation. The following plasmids were used in this study: pBR 328

(7), pUC 9 (8) and pzk 5 (9).

2.2. Chemicals

[¹⁴C]-galactose for galactokinase assay and [³⁵S]-dATP for DNA sequencing were obtained from Amersham and New England Nuclear, respectively. The oligopeptide ser-his-asp was purchased from Serva. All other chemicals were from Sigma Chemicals.

2.3. Enzymes

Restriction endonucleases and enzymes used in DNA cloning were obtained from Boehringer Mannheim. Enzyme reactions were buffered as recommended by the manufacturer and performed as described (10).

Galactokinase (*E. coli*) was purified according to a published procedure (11)

2.4. Construction of the esterase gene

Three chemically synthesized oligonucleotides (purchased from KabiGen AB, Stockholm, Sweden) d(GpApApGpCpGpCpApCpGpCpGpApGpCpTpTpTpTpTpCpTpTpT) (no 1), d(CpGpCpGpTpGpCpGpCpTpTpCpApApApGpApApApApGpCpT) (no 2) and d(CpGpCpGpTpGpCpGpCpTpTpCpTpGpCpA) (no 3), were used to build up the esterase gene. Each oligonucleotide (1 µg) was phosphorylated in a 10 µl reaction mixture with T4 polynucleotide kinase. After 60 minutes at 37 °C the three oligonucleotides were mixed in the ratio of No 1:No 2:No 3 as 100:100:1, heated to 60 °C for 5 minutes and then slowly cooled to room temperature to allow selfhybridization. T4 DNA ligase was added and the mixture was incubated overnight at room temperature. The polymers were concentrated by ethanol precipitation. Finally, approximately 0.5 µg of the polymer mixture was treated with the Klenow fragment for 15 minutes to produce DNA fragments with a PstI site at one end and a blunt end at the other (figure 1). This mixture was directly ligated to pBR 328 earlier digested with PstI and PvuII. The nucleotide sequences of a few of these DNA fragments were confirmed by the Sanger dideoxy chain termination method (12). The esterase genes were transferred from pBR 328 to pUC 9 as PstI/EcoRI fragments. To one of the obtained plasmids, pES 7, carrying seven esterase units, the galK gene was joined as an EcoRI

fragment to the 3'-end of the esterase gene. The resulting plasmid, pES 27, codes for an in frame fusion between the esterase and the galactokinase. The small fragment (100 bp) between the PvuII and EcoRI sites in pBR 328 was used as a linker region between the two catalytic entities (figure 2).

2.5. Enzyme assays and protein determination

Galactokinase activity was determined using [^{14}C]-galactose (13). One unit of enzyme phosphorylates 1 μmole of galactose per minute at 30 °C.

Esterase activity was assayed by following the hydrolysis of p-nitrophenyl esters spectrophotometrically at 405 nm. The substrates, p-nitrophenyl acetate, propionate, butyrate, caproate and caprylate, were dissolved in acetonitrile. Subsequently, ethanol and 0.07 M potassium phosphate buffer pH 7.8 were added to a final composition of solution of 1:4:95 of acetonitrile: ethanol:buffer, respectively. All measurements were carried out under first order reaction conditions, i.e. with the catalyst in excess of ester. Substrate and catalyst concentrations were 0.1 mM and 0.2 mM, respectively.

During protein purification p-nitrophenyl butyrate was used as substrate. This compound showed greater stability in the presence of interfering enzymes in an E. coli homogenate as compared to p-nitrophenyl acetate. Thus, one unit of esterase was defined as a change of one A unit/min at 21-22 °C using p-nitrophenyl butyrate as substrate.

Protein concentrations were determined using the Bradford method (14).

2.6. Protein purification

All operations were performed at 0-4 °C.

Preparation of extract: The hybrid protein was purified from E. coli C600 K⁻ carrying pES 27 grown to middle exponential phase ($\text{OD}_{550} = 2.5$) in LB medium containing 100 mg/l of ampicillin. The cells were harvested by centrifugation at 9,000 x g and washed once with chilled 0.005 M sodium phosphate buffer at pH 7.0. Ordinarily 1 liter yielded 4 to 5 grams of packed cells.

The cells were suspended in 4 volumes of 0.02 M sodium phosphate buffer containing 10 mM mercaptoacetic acid and 1mM EDTA at a final pH of 7.0 and then sonicated for 5 minutes in a sonic oscillator (Sonifier B-30, Branson Sonic Power). The suspension was clarified by centrifugation at 20,000 x g for 10 minutes.

Ammonium sulfate precipitation: The suspension was made 50 % saturated with respect to ammonium sulfate by slow addition of solid $(\text{NH}_4)_2\text{SO}_4$. After 30 minutes the precipitate was removed by centrifugation at 20,000 x g for 30 minutes and suspended in a minimum volume of 0.1 M sodium phosphate buffer pH 7.0 containing 1 mM EDTA and 10 mM mercaptoacetic acid (Buffer A).

Sephacryl S-400 Superfine gel filtration: The protein solution was subjected to chromatography on a Sephacryl S-400 SF column (97x2.5 cm) equilibrated with buffer A. Fractions containing both galactokinase and esterase activities were concentrated by ammonium sulfate precipitation. The sample was dissolved in 0.02 M sodium phosphate pH 7.4 containing 1 mM EDTA and 10 mM mercaptoacetic acid (Buffer B) and dialyzed against the same buffer.

DEAE-Sephacel Fast Flow: The sample was clarified by centrifugation for 30 minutes at 20,000 x g and the supernatant was applied to DEAE-Sephacel earlier equilibrated with buffer B. The column was washed with 5-10 volumes of the same buffer and the hybrid protein was eluted with a linear gradient of 0-300 mM NaCl.

Phenyl-Sepharose: NaCl was added to the esterase-galactokinase containing fractions to a final concentration of 2 M. The sample was adsorbed on a Phenyl-Sepharose column (2 ml) and was washed with 10 volumes of Buffer B containing 2 M NaCl and then with 10 volumes of buffer B containing 0.5 M NaCl. The hybrid protein was eluted with water.

3. RESULTS

3.1. Purification of hybrid protein

During initial work on purification of the synthetic esterase it was found that an essential

prerequisite for successful isolation of the artificial polypeptide was the fusion to a "marker" enzyme which also could serve as an "affinity tail" during protein purification. In addition, the molecular weight was increased which is highly favourable since there were several low molecular weight proteins exhibiting esterase activity and thus "masking" the esterase mimic during e.g. gel filtration. Galactokinase has been used for gene fusion purposes earlier (9,18). It is a small monomeric enzyme ($M_w = 40,000$) and it shows a low intrinsic esterase activity. Thus, by taking advantage of both enzymic activities we were able to purify the hybrid protein using conventional chromatographic procedures: ammonium sulfate precipitation, gel filtration (figure 3), ion exchange (figure 4) and hydrophobic chromatography.

As judged from sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis this resulted in a homogeneous preparation (figure 5). According to SDS-PAGE and gel filtration the protein was monomeric since both analyses indicated a molecular weight of 51,000-53,000. This was in agreement with the theoretically calculated value of 52,000. The esterase-galactokinase fusion protein showed specific activities of 60 U/mg and 0.45 U/mg for the galactokinase and esterase moieties, respectively.

3.2. Catalytic properties of the hybrid protein

The catalytic properties of the bifunctional enzyme were evaluated by determining the rate of hydrolysis of p-nitrophenyl esters of acetic, propionic, butyric, caproic and caprylic acid. The observed rate constants were compared with those of naturally occurring esterases. Table 1 gives the relative rate of hydrolysis of p-nitrophenyl acetate for a variety of catalysts, both amino acids and peptides, where equimolar concentration of each esterase has been used. The catalytic activity of histidine has been used as reference. The data presented suggest that there is a cooperative effect between the functional groups of an oligo- or polypeptide compared with free amino acids since the latter, alone or in a mixture with other free amino acids, show a much lower relative activity.

To evaluate the influence of the galactokinase part of the hybrid enzyme on the esterase

activity, the purified enzyme was subjected to proteolytic degradation by trypsin immobilized to tressyl chloride activated Sepharose 4B (15). This resulted in a total degradation of both the galactokinase and the spacer but left the esterase intact since the latter has no lysine or arginine residue. The catalytic capacity was reduced by 9 % suggesting that the effect of the galactokinase moiety on the esterase was negligible. In addition, native galactokinase as such showed a low intrinsic esterase activity.

To examine the effect of the phe-phe-phe groups which were introduced as substrate binding sites in analogy to Nilsson and Mosbach (3), the catalytic activity was tested on p-nitrophenyl esters of carboxyl acids with different lengths of the aliphatic chain. As can be seen from figure 6 the esterase exhibited a higher relative catalytic activity against more hydrophobic substrates as compared with mono histidine or ser-his-asp, thus indicating that the hydrophobic substrates interacted more favourably with the hybrid protein.

4. DISCUSSION

In this study we have made an attempt to design an enzyme-like catalyst from the "ground up"(16). We have chosen to prepare an esterase/protease mimic since the catalytic mechanism is known for many of these hydrolytic enymes. When studying naturally occurring enzymes, the polypeptide chain might be looked upon as composed of a substrate binding site and a catalytic site. Therefore, it seems as an essential feature of an enzyme designed de novo to have both of these sites in a final construction. Starting with the catalytic site, ester hydrolysis can be based upon monofunctional catalysis using imidazolyl groups, but much improved catalysis is obtained if different active groups are cooperating. Furthermore, these groups should have an optimal internal environment. However, it is not possible to predict the tertiary structure of a protein from its amino acid sequence alone and thus a prediction on the catalytic efficiency is precluded. Nevertheless, by creating a long flexible polypeptide chain carrying several repetitive catalytic groups some of them might obtain suitable interactions for catalysis by a random folding process. Glutamic acid was chosen instead of aspartic acid due to give increased flexibilty to the polypeptide and to permit the

catalytic groups of the artificial esterase to interact with each other in a similar mode as the "charge-relay system" of chymotrypsin (17). When the catalytic efficiency of the esterase is compared with e.g. ser-his-asp it seems plausible that such favourable interactions at least take place in part, but the individual role of these functional groups has to be evaluated by e.g. selective chemical modification. This comparison is based only on the release of p-nitrophenol in the described ester hydrolysis. The deacylation step, necessary for turnover, has not been evaluated. A detailed kinetic analysis will be carried out later.

In addition to the catalytic activity of the polypeptide, the substrate binding region is important. Since the conformation of the artificial polypeptide is unknown, it is difficult to design a binding site, but by simply introducing hydrophobic regions, phe-phe-phe, in between the catalytic groups a stronger affinity for hydrophobic substrates was obtained as compared to mono histidine or ser-his-asp.

Since the knowledge of protein folding and enzyme mechanism continues to advance more intelligent conclusions can be drawn of how to construct improved synthetic catalysts employing e.g. computer graphics. The use of genetic engineering techniques to verify such theoretical assumptions is highly advantageous because of the ease by which synthetic oligonucleotides are made and subsequently expressed in high amounts in microorganisms. The described synthetic esterase catalyzes only hydrolysis of activated esters and as such is to be considered only as a first step towards the preparation of artificial enzyme-like catalysts. One of the main purposes of this study has been to focus attention to the genetic engineering approach as an alternative to the previously described purely chemical procedures. We are convinced that artificial enzyme-like catalysts produced by biological means will find widespread use in many areas in the future. Certainly, practical applications will be found in bioorganic synthesis of esters by reversal of the described ester hydrolysis and in various bioanalytical procedures.

ACKNOWLEDGEMENTS

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Table 1. Hydrolysis of p-nitrophenyl acetate

Catalyst	Relative catalytic activity
histidine (his)	1.0
glutamic acid (glu)	0.02
aspartic acid (asp)	0.004
serine (ser)	0.05
ser + his + asp	1.6
ser-his-asp	3.0
esterase-galactokinase	3,200
galactokinase	46
chymotrypsin	2,300*
papain	230,000

* p-nitrophenyl acetate is not a suitable substrate for chymotrypsin.

FIGURE LEGENDS

Figure 1. Nucleotide sequence of a segment of the cloned esterase gene, the catalytic unit, showing how it is constructed from three chemically synthesized oligonucleotides.

Figure 2. Construction of the esterase-galactokinase fusion vector.

Figure 3. Fractionation of esterase-galactokinase on Sephacryl S-400 SF (flow rate: 0.33 ml/min., fraction volume: 6.6 ml).

Figure 4. Fractionation of esterase-galactokinase on DEAE-Sephacel (flow rate: 0.43 ml/min., fraction volume: 8.6 ml).

Figure 5. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis of the esterase-galactokinase fusion protein on a 10 % acrylamide gel.

I. Purified native galactokinase.

II. Purified esterase-galactokinase.

III. Crude extract of *E. coli* C 600 K⁻ carrying pES 27.

Figure 6. Relative rate of hydrolysis of p-nitrophenyl esters of acetic acid (n=1), propionic acid (n=2), butyric acid (n=3), caproic acid (n=5) and caprylic acid (n=7) using different catalysts: histidine (---), ser-his-asp (- - -) and esterase-galactokinase (—).

Fig. 1

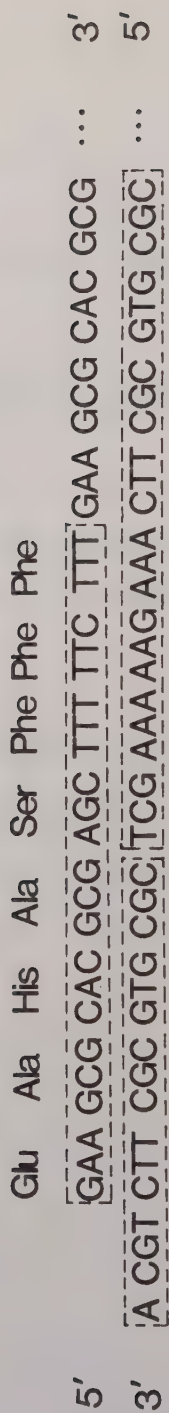


Fig. 2

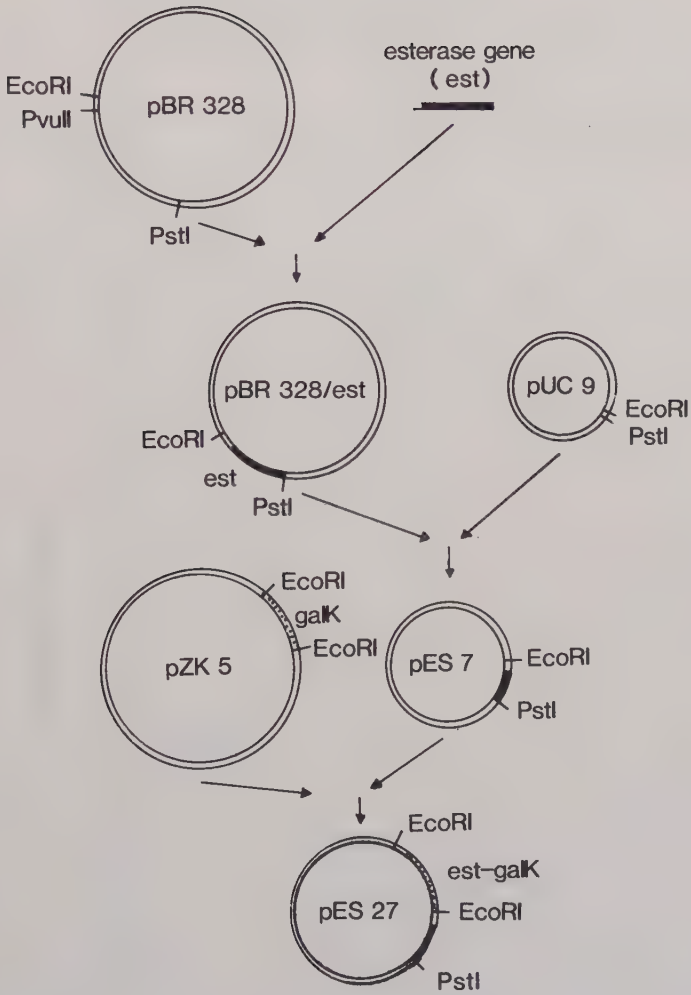


Fig. 3

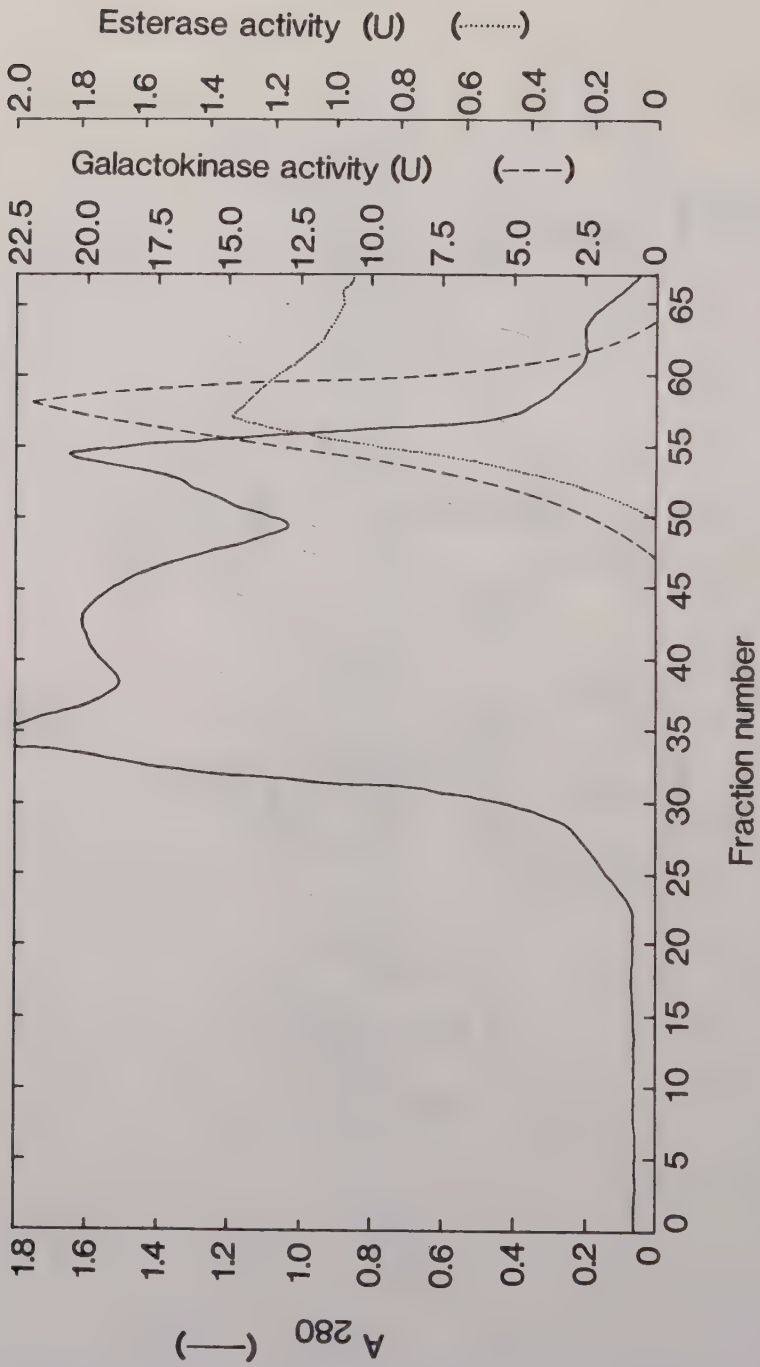


Fig. 4

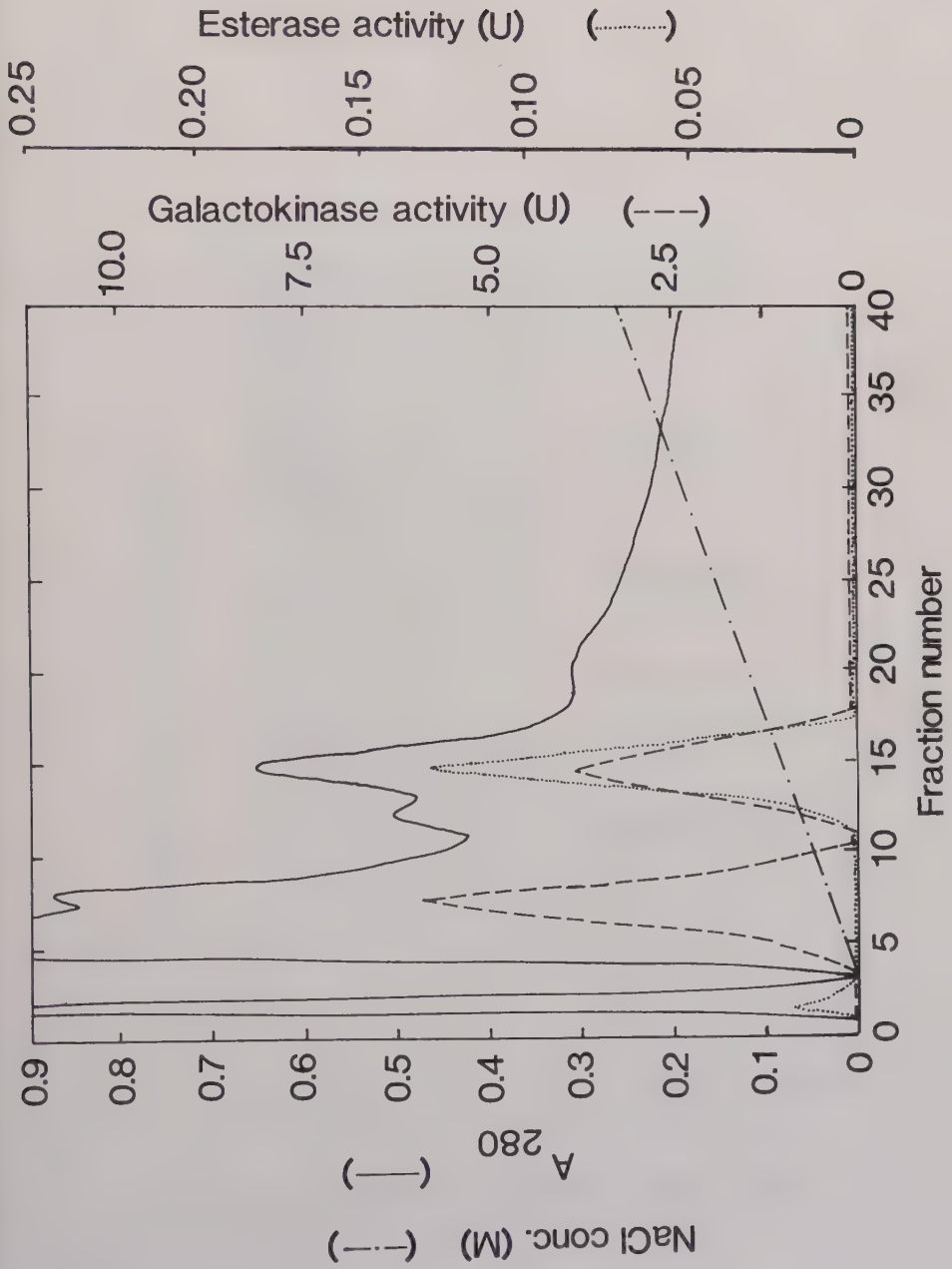


Fig. 5

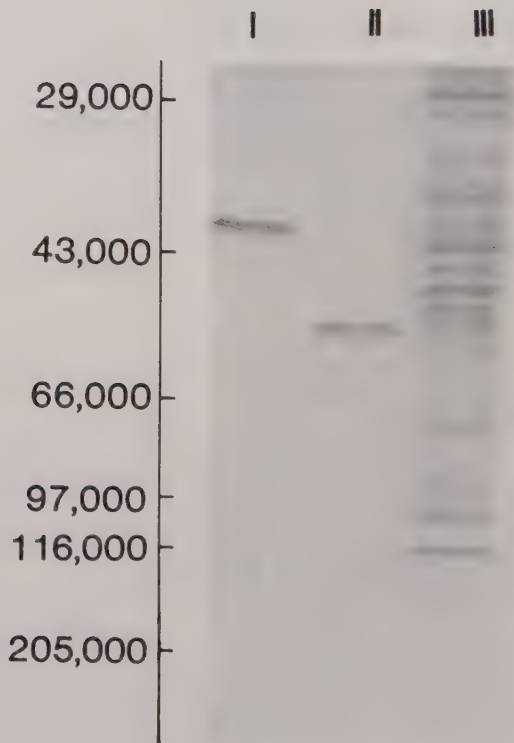


Fig. 6

